

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property  
Organization  
International Bureau



(43) International Publication Date  
11 March 2004 (11.03.2004)

PCT

(10) International Publication Number  
**WO 2004/020468 A2**

(51) International Patent Classification<sup>7</sup>: **C07K 14/555**

(21) International Application Number:

PCT/DK2003/000554

(22) International Filing Date: 25 August 2003 (25.08.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:  
60/407,188 28 August 2002 (28.08.2002) US

(71) Applicant (for all designated States except US): MAXY-  
GEN ApS [DK/DK]; Agern Allé 1, DK-2970 Hørsholm  
(DK).

(72) Inventor; and

(75) Inventor/Applicant (for US only): SØRENSEN, Poul  
[DK/DE]; c/o Micromet AG, Staffelseest. 2, D-81477  
Munich (DE).

(74) Agent: MAXYGEN ApS; Agern Allé 1, DK-2970 Hør-  
sholm (DK).

(81) Designated States (national): AE, AG, AL, AM, AT, AU,  
AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU,  
CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,  
GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC,  
LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW,  
MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC,  
SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA,  
UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (regional): ARIPO patent (GH, GM,  
KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW),  
Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),  
European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE,  
ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO,  
SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM,  
GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

**Published:**

— without international search report and to be republished  
upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guid-  
ance Notes on Codes and Abbreviations" appearing at the begin-  
ning of each regular issue of the PCT Gazette.

(54) Title: INTERFERON BETA-LIKE MOLECULES FOR TREATMENT OF CANCER

(57) Abstract: The present invention relates to interferon  $\beta$  (IFNB) polypeptides with an increased half-life in the treatment of various cancers, in particular solid tumors. More particularly, the present invention is directed to a method of treating a mammal, in particular a human being, having a cancer, wherein said cancer has malignant cells carrying interferon type 1 deletions, by administering to said mammal a therapeutically effective dose of an IFNB polypeptide with increased functional *in vivo* half-life as compared to interferon  $\beta$  1a. The present invention is also directed to a method of treating a mammal, in particular a human being, having cancer, comprising administering to said mammal a therapeutically effective dose of an interferon beta (IFNB) polypeptide with increased functional *in vivo* half-life as compared to interferon  $\beta$  1a in combination with a chemotherapeutic agent. IFNB polypeptides with increased functional *in vivo* half-life are typically obtained through PEGylation of the IFNB polypeptide.

**BEST AVAILABLE COPY**

## INTERFERON BETA-LIKE MOLECULES FOR TREATMENT OF CANCER

### FIELD OF THE INVENTION

The present invention relates to interferon  $\beta$  (IFNB) polypeptides with an increased half-life in the treatment of various cancers.

### BACKGROUND OF THE INVENTION

Interferons are important cytokines characterized by antiviral, antiproliferative, anti-angiogenic, and immunomodulatory activities. These activities form a basis for the clinical benefits that have been observed in a number of diseases, including hepatitis, various cancers, autoimmune diseases and multiple sclerosis. The interferons are divided into the type I and type II classes. IFNB belongs to the class of type I interferons, which also includes interferons  $\alpha$ ,  $\tau$  and  $\omega$ , whereas interferon  $\gamma$  is the only known member of the distinct type II class.

Human IFNB is a regulatory polypeptide with a molecular weight of 22 kDa consisting of 166 amino acid residues. It can be produced by most cells in the body, in particular fibroblasts, in response to viral infection or exposure to other biologics. It binds to a multimeric cell surface receptor, and productive receptor binding results in a cascade of intracellular events leading to the expression of IFNB inducible genes which in turn produces effects which can be classified as antiviral, antiproliferative and immunomodulatory.

The amino acid sequence of human IFNB was reported by Taniguchi, *Gene* 10:11-15, 1980, and in EP 0 083 069, EP 0 041 313 and US 4,686,191.

Crystal structures have been reported for human and murine IFNB, respectively (*Proc. Natl. Acad. Sci. USA* 94:11813-11818, 1997; *J. Mol. Biol.* 253:187-207, 1995). They have been reviewed in *Cell Mol. Life Sci.* 54:1203-1206, 1998.

Expression of IFNB in CHO cells has been reported (US 4,966,843, US 5,376,567 and US 5,795,779).

Various references disclose modification of polypeptides by polymer conjugation or glycosylation. Polymer modification of native IFNB or a C17S variant thereof has been reported (EP 0 229 108, US 5,382,657, EP 0 593 868, US 4,917,888 and WO 99/55377). US 4,904,584 discloses PEGylated lysine depleted polypeptides, wherein at least one lysine residue has been deleted or replaced with any other amino acid residue. WO 99/67291 discloses a process for conjugating a protein with PEG, wherein at least one amino acid residue on the protein is deleted and the protein is contacted with PEG under conditions sufficient to achieve

conjugation to the protein. WO 99/03887 discloses PEGylated variants of polypeptides belonging to the growth hormone superfamily, wherein a cysteine residue has been substituted with a non-essential amino acid residue located in a specified region of the polypeptide. IFNB is mentioned as one example of a polypeptide belonging to the growth hormone superfamily. WO 00/23114 discloses glycosylated and pegylated IFNB. WO 00/23472 discloses IFNB fusion proteins. WO 00/26354 discloses a method of producing a glycosylated polypeptide variant with reduced allergenicity, which as compared to a corresponding parent polypeptide comprises at least one additional glycosylation site. US 5,218,092 discloses modification of granulocyte colony stimulating factor (G-CSF) and other polypeptides so as to introduce at least one additional carbohydrate chain as compared to the native polypeptide. IFNB is mentioned as one example among many polypeptides that allegedly can be modified according to the technology described in US 5,218,092. WO 01/15736 and WO 02/074806 disclose glycosylated and PEGylated IFNB variants. Pharmaceutical formulations of various IFNB polypeptides are disclosed in WO 03/002152.

Commercial preparations of IFNB are sold under the names Betaseron® (also termed interferon  $\beta$ 1b, which is non-glycosylated, produced using recombinant bacterial cells, has a deletion of the N-terminal methionine residue and the C17S mutation), Avonex® and Rebif® (also termed interferon  $\beta$ 1a, which is glycosylated and produced by using recombinant mammalian cells) for treatment of patients with multiple sclerosis. They have shown to be effective in reducing the exacerbation rate, and more patients remain exacerbation-free for prolonged periods of time as compared with placebo-treated patients. Furthermore, the accumulation rate of disability is reduced (*Neurol.* 51:682-689, 1998).

Various side effects are associated with the use of current preparations of IFNB, including injection site reactions, fever, chills, myalgias, arthralgias, and other flu-like symptoms (*Clin. Therapeutics*, 19:883-893, 1997).

In addition, 6-40% of patients develop neutralizing antibodies to IFNB (*Int. Arch. Allergy Immunol.* 118:368-371, 1999). It has been shown that development of IFNB-neutralizing antibodies decreases the biological response to IFNB, and causes a trend towards decreased treatment effect (*Neurol.* 50:1266-1272, 1998). Neutralizing antibodies are also likely to impede the therapeutic utility of IFNB in connection with treatment of other diseases (*Immunol. Immunother.* 39:263-268, 1994).

Interferons have been shown to induce remissions in a variety of neoplastic diseases and are also important for in the physiological regulation of proliferation and differentiation of cells.

In 78% of human carcinomas, including melanomas, a deletion of the short arm of chromosome 9 (9p22) where the type 1 interferon genes are located have been found (Giavizzi et al., *European Journal of Cancer* 36, 1913-1918, 2000; Mead et al., *Cancer Research* 54, 2307-2309, May 1, 1994; Strissel et al., *Genomics* 47, 217-229, 1998).

Also in lymphocytic leukaemia a deletion of this region has been found (Heyman et al., *Int. J. Cancer* 54, 748-753, 1993; Strissel et al., *Genomics* 47, 217-229, 1998).

Without being bound by a particular theory we believe that interferons act as tumor-suppressor genes, and that loss of type 1 interferon genes is important for malignant transformation. Therefore, patients whose malignant cells carry type 1 interferon deletions, but are still sensitive to exogenous interferon could represent a subgroup with greater likelihood of responding to interferon therapy. This sub-group of patients could be selected for clinical testing by use of cytogenetic studies such as the restriction fragment length polymorphism (RFLP) method (Heyman et al., *Int. J. Cancer* 54, 748-753, 1993), or single nucleotide polymorphism (SNP) genotyping (Patil, N. et al. *Science* 294: 1719-1723, 2001).

#### **BRIEF DISCLOSURE OF THE INVENTION**

Accordingly, in a first aspect the invention relates to the use of an interferon  $\beta$  (IFNB) polypeptide with an increased functional *in vivo* half-life as compared to interferon  $\beta$ 1a for the manufacture of a medicament for the treatment of a cancer having malignant cells carrying interferon type 1 deletions.

In a second aspect the invention relates to the use of an interferon  $\beta$  (IFNB) polypeptide with an increased functional *in vivo* half-life as compared to interferon  $\beta$ 1a in combination with a chemotherapeutic agent for the manufacture of a medicament for the treatment of cancer.

In a further aspect the invention relates to a method of treating a mammal, in particular a human being, having a cancer, wherein said cancer has malignant cells carrying interferon type 1 deletions, said method comprising administering to said mammal a therapeutically effective dose of an interferon beta (IFNB) polypeptide with increased functional *in vivo* half-life as compared to interferon  $\beta$ 1a.

In an even further aspect the invention relates to a method of treating a mammal, in particular a human being, having cancer, said method comprising administering to said mammal a therapeutically effective dose of an interferon beta (IFNB) polypeptide with increased functional *in vivo* half-life as compared to interferon  $\beta$ 1a in combination with a chemotherapeutic agent.

In a still further aspect the invention relates to a pharmaceutical composition comprising

- i) an interferon beta (IFNB) polypeptide with increased functional *in vivo* half-life as compared to interferon  $\beta$ 1a;
- ii) a chemotherapeutic agent; and
- iii) a pharmaceutically acceptable diluent, carrier or adjuvant.

## BRIEF DISCLOSURE OF THE DRAWINGS

Fig. 1 shows mean normalized tumor volume curves of A375.S2 human malignant melanoma xenografts grown in nude mice. Arrows indicate days of treatment. ♦: pre-treatment; ■: Controls; ×: Rebif® (1.2 MIU/ml); ▲: 20kDa mono-pegylated [C17S+Q49N+Q51T+D110F+F111N+R113T]hIFN- $\beta$  (0.25 MIU/ml); ●: 20kDa mono-pegylated [C17S+Q49N+Q51T+D110F+F111N+R113T]hIFN- $\beta$  (1.5 MIU/ml).

## DETAILED DISCLOSURE OF THE INVENTION

In the present application a number of references are referred to. They are all intended to be incorporated herein by reference.

### Definitions

In the context of the present application and invention the following definitions apply:

The term "solid malignant tumor" is intended to indicate an abnormal malignant mass of tissue that is not inflammatory, which arises without obvious cause from cells of preexistent tissue, which possesses no physiologic function and which has the ability to invade normal cells and spread throughout the body. Examples of typical solid malignant tumors include breast carcinoma, non-small cell lung cancer, colon carcinoma, renal cell carcinoma and malignant melanoma.

The term "carcinoma" is intended to indicate a malignant tumor of epithelial origin. Epithelial tissue covers or lines the body surfaces inside and outside the body. Examples of epithelial tissue are the skin and the mucosa and serosa that line the body cavities and internal organs, such as intestines, urinary bladder, uterus, etc. Epithelial tissue may also extend into deeper tissue layers to form glands, such as mucus-secreting glands.

The term "sarcoma" is intended to indicate a malignant tumor growing from connective tissue, such as cartilage, fat, muscles, tendons and bones.

The term "leukaemia" is intended to indicate a blood cancer, i.e. a cancer that originates from the bone marrow and which keeps the marrow from producing normal red and white blood cells and platelets.

The term "lymphoma" refers to a cancer that originates in the nodes or glands of the lymphatic system.

The term "glioma", when used herein, is intended to cover a malignant tumor originating from glial cells.

The term "inhibition" or "inhibit" as used in connection with treatment of solid tumors is intended to cover the delayed appearance of primary or secondary tumors, slowed development of primary or secondary tumors, decreased occurrence of primary or secondary tumors, slowed or decreased severity of secondary effects of disease, arrested tumor growth and regression of tumors. The term "reduction" or "reducing" in connection with tumor size is covered by the term "inhibition" or "inhibit". The reduction of the solid tumor refers to a reduction of the tumor volume. For example, a 10% reduction of a solid tumor means that the volume of the treated tumor has been reduced with 10%.

The term "conjugate" (or interchangeably "conjugated polypeptide") is intended to indicate a heterogeneous (in the sense of composite or chimeric) molecule formed by the covalent attachment of one or more polypeptide(s) to one or more non-polypeptide moieties. The term covalent attachment means that the polypeptide and the non-polypeptide moiety are either directly covalently joined to one another, or else are indirectly covalently joined to one another through an intervening moiety or moieties, such as a bridge, spacer, or linkage moiety or moieties using an attachment group present in the polypeptide. Preferably, the conjugate is soluble at relevant concentrations and conditions, i.e. soluble in physiological fluids such as blood. Examples of conjugated polypeptides for use in the invention include glycosylated and/or PEGylated polypeptides. The term "non-conjugated polypeptide" may be used about the polypeptide part of the conjugate.

The term "non-polypeptide moiety" is intended to indicate a molecule that is capable of conjugating to an attachment group of a polypeptide for use in the invention. Preferred examples of such molecules include polymer molecules, sugar moieties, lipophilic compounds, or organic derivatizing agents. When used in the context of a conjugate as described herein it will be understood that the non-polypeptide moiety is linked to the polypeptide part of the conjugate through an attachment group of the polypeptide.

The term "polymer molecule" is defined as a molecule formed by covalent linkage of two or more monomers, wherein none of the monomers is an amino acid residue, except where the polymer is human albumin or another abundant plasma protein. The term "polymer" may be used interchangeably with the term "polymer molecule". Examples of preferred polymer molecules include PEG, such as mPEG. The term "polymer molecule" is also intended to cover carbohydrate molecules attached by *in vitro* glycosylation, i.e. synthetic glycosylation performed *in vitro* normally involving covalently linking a carbohydrate molecule to an attachment group of the polypeptide, optionally using a cross-linking agent.

Carbohydrate molecules attached by *in vivo* glycosylation, such as N- or O-glycosylation (as described further below) are referred to herein as "a sugar moiety". Normally, the *in vivo* glycosylation site is an N-glycosylation site, but also an O-glycosylation site is contemplated as relevant for the present invention. It will be understood that a glycosylated IFNB variant may also be termed an IFNB conjugate (comprising a non-polypeptide moiety being a sugar moiety attached to the polypeptide part of the conjugate).

Except where the number of non-polypeptide moieties, such as polymer molecule(s) or sugar moieties in the conjugate is expressly indicated every reference to "a non-polypeptide moiety" contained in a conjugate or otherwise used herein shall be a reference to one or more non-polypeptide moieties, such as polymer molecule(s) or sugar moieties, in the conjugate.

The term "attachment group" is intended to indicate an amino acid residue group of the polypeptide capable of coupling to the relevant non-polypeptide moiety. For instance, for a polymer, in particular PEG, a frequently used attachment group is the  $\epsilon$ -amino group of lysine or the N-terminal amino group. Other polymer attachment groups include a free carboxylic acid group (e.g. that of the C-terminal amino acid residue or of an aspartic acid or glutamic acid residue), suitably activated carbonyl groups, mercapto groups (e.g. that of a cysteine residue), aromatic acid residues (e.g. Phe, Tyr, Trp), hydroxy groups (e.g. that of Ser, Thr or OH-Lys), guanidine (e.g. Arg), imidazole (e.g. His), and oxidized carbohydrate moieties.

For *in vivo* N-glycosylation, the term "attachment group" is used in an unconventional way to indicate the amino acid residues constituting an N-glycosylation site (with the sequence N-X'-S/T/C-X'', wherein X' is any amino acid residue except proline, X'' any amino acid residue that may or may not be identical to X' and preferably is different from proline, N is asparagine and S/T/C is either serine, threonine or cysteine, preferably serine or threonine, and most preferably threonine). Although the asparagine residue of the N-glycosylation site is the one to which the sugar moiety is attached during glycosylation, such attachment cannot be

achieved unless the other amino acid residues of the N-glycosylation site is present. Accordingly, when the non-polypeptide moiety is an N-linked sugar moiety, the term "amino acid residue comprising an attachment group for the non-polypeptide moiety" as used in connection with alterations of the amino acid sequence of the parent polypeptide is to be understood as amino acid residues constituting an N-glycosylation site is/are to be altered in such a manner that a functional N-glycosylation site is introduced into the amino acid sequence. For an "O-glycosylation site" the attachment group is the OH-group of a serine or threonine residue.

It should be understood that when the term "at least 25% (or 50%) of its side chain exposed to the surface" is used in connection with introduction of an *in vivo* N-glycosylation site this term refers to the surface accessibility of the amino acid side chain in the position where the sugar moiety is actually attached. In many cases it will be necessary to introduce a serine or a threonine residue in position +2 relative to the asparagine residue to which the sugar moiety is actually attached and these positions, where the serine or threonine residues are introduced, are allowed to be buried, i.e. to have less than 25% (or 50%) of their side chains exposed to the surface.

The sugar moiety attached to a glycosylation site is typically sialylated. However, the sialic acid may be removed, e.g. by enzymatic cleavage by neuraminidase, to produce an asialo-glycosylated IFNB polypeptide (Brady et al. *J. Inher. Metab. Dis.* (1994) 17, 510-519 and US 5,549,892). In another embodiment, the sugar moieties are further modified to contain only mannose. This may be done by sequential treatment with neuraminidase,  $\beta$ -galactosidase and  $\beta$ -N-acetylglucosaminidase (Brady et al. *J. Inher. Metab. Dis.* (1994) 17, 510-519 and US 5,549,892).

The term "amino acid residue comprising an attachment group for the non-polypeptide moiety" is intended to indicate that the amino acid residue is one to which the non-polypeptide moiety binds (in the case of an introduced amino acid residue) or would have bound (in the case of a removed amino acid residue).

The term "one difference" or "differs from" as used in connection with specific modifications, such as substitution, is intended to allow for additional differences being present apart from the specified amino acid difference. For instance, in addition to the removal and/or introduction of amino acid residues comprising an attachment group for the non-polypeptide moiety the IFNB polypeptide may comprise other substitutions that are not related to introduction and/or removal of such amino acid residues. These may, for example, include



truncation of the C-terminus by one or more amino acid residues, truncation of the N-terminus by one or more amino acid residues and/or "conservative amino acid substitutions", i.e. substitutions performed within groups of amino acids with similar characteristics, e.g. small amino acids, acidic amino acids, polar amino acids, basic amino acids, hydrophobic amino acids and aromatic amino acids. Examples of conservative substitutions in the present invention may in particular be selected from the groups listed in the table below.

1	Alanine (A)	Glycine (G)	Serine (S)	Threonine (T)
2	Aspartic acid (D)	Glutamic acid (E)		
3	Asparagine (N)	Glutamine (Q)		
4	Arginine (R)	Histidine (H)	Lysine (K)	
5	Isoleucine (I)	Leucine (L)	Methionine (M)	Valine (V)
6	Phenylalanine (F)	Tyrosine (Y)	Tryptophan (W)	

The term "a" as used about a non-polypeptide moiety, an amino acid residue, a substitution, etc. is intended to mean one or more. In particular, the expression "a component (such as a substitution, an amino acid residue or the like) selected from the group consisting of ..." is intended to mean that one or more of the cited components may be selected. Thus, expressions like "a component selected from the group consisting of A, B and C" is intended to include all combinations of A, B and C, i.e. A, B, C, A+B, A+C, B+C or A+B+C.

The term "at least one" as used about a non-polypeptide moiety, an amino acid residue, a substitution, etc. is intended to mean one or more.

In the present application, amino acid names and atom names (e.g. CA, CB, CD, CG, SG, NZ, N, O, C, etc) are used as defined by the Protein DataBank (PDB) ([www.pdb.org](http://www.pdb.org)) which are based on the IUPAC nomenclature (IUPAC Nomenclature and Symbolism for Amino Acids and Peptides (residue names, atom names e.t.c.), *Eur. J. Biochem.*, 138, 9-37 (1984) together with their corrections in *Eur. J. Biochem.*, 152, 1 (1985). CA is sometimes referred to as C $\alpha$ , CB as C $\beta$ . The term "amino acid residue" is intended to indicate an amino acid residue contained in the group consisting of alanine (Ala or A), cysteine (Cys or C), aspartic acid (Asp or D), glutamic acid (Glu or E), phenylalanine (Phe or F), glycine (Gly or G), histidine (His or H), isoleucine (Ile or I), lysine (Lys or K), leucine (Leu or L), methionine (Met or M), asparagine (Asn or N), proline (Pro or P), glutamine (Gln or Q), arginine (Arg or R), serine (Ser or S), threonine (Thr or T), valine (Val or V), tryptophan (Trp or W), and tyrosine

(Tyr or Y) residues. The terminology used for identifying amino acid positions/substitutions is illustrated as follows:

The terminology used for identifying amino acid positions/substitutions is illustrated as follows: C17 indicates that position 17 is occupied by a cysteine residue in the amino acid sequence shown in SEQ ID NO 2. C17S indicates that the Cys residue of position 17 has been replaced with a Ser residue. Multiple substitutions are indicated with a "+", e.g. R71N+D73T/S means an amino acid sequence which comprises a substitution of the Arg residue in position 71 with an Asn residue and a substitution of the Asp residue in position 73 with a Thr or Ser residue, preferably a Thr residue. T/S as used about a given substitution herein means either a T or S residue, preferably a T residue. Deletions are indicated by an asterix. For example, M1\* indicates that the Met residue in position 1 has been deleted. Insertions are indicated the following way: Insertion of an additional Phe residue after the Cys residue located in position 17 is indicated as C17CF. Combined substitutions and insertions are indicated in the following way: Substitution of the Cys residue at position 17 with an Ser residue and insertion of a Phe residue after the position 17 amino acid residue is indicated as C17SF.

The term "nucleotide sequence" is intended to indicate a consecutive stretch of two or more nucleotide molecules. The nucleotide sequence may be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any combinations thereof.

The term "IFNB protein sequence family" is used in its conventional meaning, i.e. to indicate a group of polypeptides with sufficiently homologous amino acid sequences to allow alignment of the sequences, e.g. using the CLUSTALW program. An IFNB sequence family is available, e.g. from the PFAM families, version 4.0, or may be prepared by use of a suitable computer program such as CLUSTALW version 1.74 using default parameters (Thompson et al., 1994, CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, *Nucleic Acids Research*, 22:4673-4680).

An "equivalent position" is intended to indicate a position in the amino acid sequence of a given IFNB polypeptide, which is homologous (i.e. corresponding in position in either primary or tertiary structure) to the relevant position in the amino acid sequence shown in SEQ ID NO 2. The "equivalent position" is conveniently determined on the basis of an alignment of members of the IFNB protein sequence family, e.g. using the CLUSTALW program described above.

"Cell", "host cell", "cell line" and "cell culture" are used interchangeably herein and all such terms should be understood to include progeny resulting from growth or culturing of a cell.

"Transformation" and "transfection" are used interchangeably to refer to the process of introducing DNA into a cell.

"Operably linked" refers to the covalent joining of two or more nucleotide sequences, by means of enzymatic ligation or otherwise, in a configuration relative to one another such that the normal function of the sequences can be performed. For example, the nucleotide sequence encoding a presequence or secretory leader is operably linked to a nucleotide sequence for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the nucleotide sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used, in conjunction with standard recombinant DNA methods.

The term "introduce" is primarily intended to mean substitution of an existing amino acid residue, but may also mean insertion of an additional amino acid residue.

The term "remove" is primarily intended to mean substitution of the amino acid residue to be removed by another amino acid residue, but may also mean deletion (without substitution) of the amino acid residue to be removed.

The term "modification", as used herein, covers substitution, insertion and deletion.

The terms "mutation" and "substitution" are used interchangeably herein.

The term "immunogenicity" as used in connection with a given substance is intended to indicate the ability of the substance to induce a response from the immune system. The immune response may be a cell or antibody mediated response (see, e.g., Roitt: Essential Immunology (8<sup>th</sup> Edition, Blackwell) for further definition of immunogenicity). Immunogenicity may be determined by use of any suitable method known in the art, e.g. *in vivo* or *in vitro*, e.g. using the *in vitro* immunogenicity test outlined in the Materials and Methods section below.

The term "reduced immunogenicity" as used about a given polypeptide or conjugate is intended to indicate that the conjugate or polypeptide gives rise to a measurably lower immune response than a reference molecule, such as wild-type human IFNB, e.g., Rebif® or Avonex®,

or a variant of wild-type human IFNB, such as Betaseron®, as determined under comparable conditions. When reference is made herein to commercially available IFNB products (i.e. Betaseron®, Avonex® and Rebif®), it should be understood to mean either the formulated product or the IFNB polypeptide part of the product (as appropriate). Normally, reduced antibody reactivity (e.g. reactivity towards antibodies present in serum from patients treated with commercial IFNB products) is an indication of reduced immunogenicity. However, antibodies present in serum from patients treated with a commercial IFNB product have shown a high degree of cross-reactivity to other IFNB products although their capacity for eliciting an antibody response may be different (*Neurology* 51:1698-1702,1998). This indicate that the true immunogenic potential of a polypeptide cannot be determined without conducting trials with "naive" subjects not previously treated with IFNB products.

The term "circulating antibodies" is intended to indicate antibodies, in particular neutralizing antibodies, formed in a mammal in response to treatment with interferon  $\beta$ 1a or  $\beta$ 1b, such as Rebif®, Betaseron® or Avonex®.

The term "functional *in vivo* half-life" is used in its normal meaning, i.e. the time at which 50% of a given functionality of the polypeptide or conjugate is retained (such as the time at which 50% of the biological activity of the polypeptide or conjugate is still present in the body/target organ, or the time at which the activity of the polypeptide or conjugate is 50% of the initial value).

As an alternative to determining functional *in vivo* half-life, "serum half-life" may be determined, i.e. the time in which 50% of the polypeptide or conjugate molecules circulate in the plasma or bloodstream prior to being cleared. Determination of serum half-life is often more simple than determining functional *in vivo* half-life and the magnitude of serum half-life is usually a good indication of the magnitude of functional *in vivo* half-life. Alternative terms to serum half-life include "plasma half-life", "circulating half-life", "serum clearance", "plasma clearance" and "clearance half-life". The functionality to be retained is normally selected from antiviral, antiproliferative, immunomodulatory or receptor binding activity. Functional *in vivo* half-life and serum half-life may be determined by any suitable method known in the art as further discussed in the Materials and Methods section hereinafter.

The polypeptide or conjugate is normally cleared by the action of one or more of the reticuloendothelial systems (RES), kidney, spleen or liver, or by specific or unspecific proteolysis. Clearance taking place by the kidneys may also be referred to as "renal clearance" and is e.g. accomplished by glomerular filtration, tubular excretion or tubular elimination.

Normally, clearance depends on physical characteristics of the polypeptide or conjugate, including molecular weight, size (diameter) (relative to the cut-off for glomerular filtration), charge, symmetry, shape/rigidity, attached carbohydrate chains, and the presence of cellular receptors for the protein. A molecular weight of about 67 kDa is considered to be an important cut-off-value for renal clearance.

Reduced renal clearance may be established by any suitable assay, e.g. an established *in vivo* assay. Typically, the renal clearance is determined by administering a labelled (e.g. radio-labelled or fluorescence-labelled) polypeptide or polypeptide conjugate to a patient and measuring the label activity in urine collected from the patient. Reduced renal clearance is determined relative to the corresponding non-conjugated polypeptide or the non-conjugated corresponding wild-type polypeptide or a commercial IFNB product under comparable conditions.

The term "increased" as used about the functional *in vivo* half-life or serum half-life is used to indicate that the relevant half-life of the polypeptide or conjugate is statistically significantly increased relative to that of interferon  $\beta 1a$  (Avonex® or Rebif®) as determined under comparable conditions.

Thus, interesting IFNG polypeptide with an increased *in vivo* functional half-life are such polypeptides where the ratio between the functional *in vivo* half-life (or serum half-life) of said IFNB polypeptide and the functional *in vivo* half-life (or serum half-life) of interferon  $\beta 1a$  is at least 1.25, more preferably at least 1.50, even more preferably at least 1.75, such as at least 2, when administered intravenously in an experimental animal, such as rats, minipigs or monkeys.

In a similar way, interesting IFNG polypeptide with an increased *in vivo* functional half-life are such polypeptides where the ratio between the functional *in vivo* half-life (or serum half-life) of said IFNB polypeptide and the functional *in vivo* half-life (or serum half-life) of interferon  $\beta 1a$  is at least 1.25, such as at least 1.50, e.g. at least 2, preferably at least 3, such as at least 4, e.g. at least 5, more preferably at least 6, such as at least 7, e.g. at least 8, even more preferably at least 9, such as at least 10, when administered intravenously in an experimental animal, such as rats, minipigs or monkeys.

The term "reduced immunogenicity and/or increased functional *in vivo* half-life and/or increased serum half-life" is to be understood as covering any one, two or all of these properties. In an interesting embodiment, a conjugate or polypeptide as described herein has at least two of these properties, i.e. reduced immunogenicity and increased functional *in vivo* half-

life, reduced immunogenicity and increased serum half-life or increased functional *in vivo* half-life and increased serum half-life.

The term "under comparable conditions" as used about measuring of relative (rather than absolute) properties of a molecule for use in the invention and a reference molecule is intended to indicate that the relevant property of the two molecules is assayed using the same assay (i.e. the assay is performed under the same conditions including the same internal standard), and, when relevant, the same type of animals.

The term "exhibiting IFNB activity" is intended to indicate that the polypeptide or conjugate has one or more of the functions of native IFNB, in particular human wild-type IFNB with the amino acid sequence shown in SEQ ID NO 2 (which is the mature sequence) optionally expressed in a glycosylating host cell, or any of the commercially available IFNB products. Such functions include capability to bind to an interferon receptor that is capable of binding IFNB and initiating intracellular signalling from the receptor, in particular a type I interferon receptor constituted by the receptor subunits IFNAR-2 and IFNAR-1 (Domanski et al., *The Journal of Biological Chemistry*, Vol. 273, No. 6, pp3144-3147, 1998, Mogensen et al., *Journal of Interferon and Cytokine Research*, 19: 1069-1098, 1999), and antiviral, antiproliferative or immunomodulatory activity (which can be determined using assays known in the art (e.g. those cited in the following disclosure)). IFNB activity may be assayed by methods known in the art as exemplified in the Materials and Methods section hereinafter.

The polypeptide or conjugate "exhibiting" or "having" IFNB activity is considered to have such activity, when it displays a measurable function, e.g. a measurable receptor binding and stimulating activity (e.g. as determined by the primary or secondary assay described in the Materials and Methods section). The polypeptide exhibiting IFNB activity may also be termed "IFNB molecule", "IFNB variant polypeptide" or "IFNB polypeptide" herein. The terms "IFNB polypeptide", "IFNB variant" and "variant polypeptide" are primarily used herein about modified polypeptides for use in the invention.

The term "parent IFNB" is intended to indicate the starting molecule to be improved for use in accordance with the present invention. Preferably, the parent IFNB belongs to the IFNB sequence family. While the parent IFNB may be of any origin, such as vertebrate or mammalian origin (e.g. any of the origins defined in WO 00/23472), the parent IFNB is preferably wild-type human IFNB with the amino acid sequence shown in SEQ ID NO 2 or a variant thereof.

In the context of a parent IFNB polypeptide, a "variant" is a polypeptide, which differs in one or more amino acid residues from a parent IFNB polypeptide, such as wild-type human IFNB. Typically, the variant differs from the parent IFNB polypeptide, such as wild-type human IFNB, in 1-15 amino acid residues, 1-10 amino acid residues, 1-8 amino acid residues, 2-8 amino acid residues, 1-5 amino acid residues or 2-5 amino acid residues. Thus, typically the variant differs from the parent IFNB polypeptide, such as wild-type human IFNB, in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14 or 15 amino acid residues. Examples of wild-type human IFNB polypeptides include the polypeptide part of Avonex® or Rebif®. An example of a parent IFNB variant is Betaseron®. Alternatively, the parent IFNB polypeptide may comprise an amino acid sequence, which is a hybrid molecule between IFNB and another homologous polypeptide, such as interferon  $\alpha$ , optionally containing one or more additional substitutions introduced into the hybrid molecule. Such a hybrid molecule may contain an amino acid sequence, which differs in more than 10 amino acid residues from the amino acid sequence shown in SEQ ID NO 2. In order to be useful as a parent polypeptide the hybrid molecule exhibits IFNB activity (e.g. as determined in the secondary assay described in the Materials and Methods section herein). Other examples of variants of wild-type human IFNB that may serve as parent IFNB molecules in the present invention are the variants described in WO 01/15736 having introduced and/or removed amino acid residues comprising an attachment group for a non-polypeptide moiety, or any of the IFNB molecules described in WO 00/23114, WO 00/23472, WO 99/3887 or otherwise available in the art.

In the present context the terms "interferon beta polypeptide", "IFNB polypeptide", "interferon beta molecule" or "IFNB molecule" cover wild-type human IFNB as well as variants thereof.

The term "interferon  $\beta$ 1a" refers to wild-type human interferon  $\beta$  having the amino acid sequence shown in SEQ ID NO 2 and which is glycosylated in position N80. Examples of interferon  $\beta$ 1a include Avonex® and Rebif®.

The terms "hIFNB" and "hIFN- $\beta$ " refer to wild-type human interferon beta having the amino acid sequence shown in SEQ ID NO 2.

The term "functional site" as used about a polypeptide or conjugate for use in the invention is intended to indicate one or more amino acid residues which is/are essential for or otherwise involved in the function or performance of IFNB, and thus "located at" the functional site. The functional site is e.g. a receptor binding site and may be determined by methods

known in the art, preferably by analysis of a structure of the polypeptide being complexed to a relevant receptor, such as the type I interferon receptor constituted by IFNAR-1 and IFNAR-2.

In the present context the term "increased glycosylation" is intended to indicate increased levels of attached carbohydrate molecules, normally obtained as a consequence of increased (or better) utilization of glycosylation site(s). The increased glycosylation may be determined by any suitable method known in the art for analyzing attached carbohydrate structures. One convenient assay for determining attached carbohydrate structures is the method described in Example 7 and 8 herein.

An amino acid residue "located close to" a glycosylation site is usually located in position -4, -3, -2, -1, +1, +2, +3 or +4 relative to the amino acid residue of the glycosylation site to which the sugar moiety is attached, in particular in position -2, -1, +1, or +2, such as position -1 or +1, in particular position -1. These positions may be modified to increase the glycosylation at the site. The modification is normally a substitution, the substitution being made with any other amino acid residue that gives rise to an increased glycosylation of the IFNB variant as compared to that of the parent IFNB polypeptide. Such other amino acid residue may be determined by trial and error type of experiments (i.e. by substitution of the amino acid residue of the relevant position to any other amino acid residue, and determination of the resulting glycosylation of the resulting variant).

When used herein the term "naturally occurring glycosylation site" is intended to mean the N-glycosylation site defined by N80 and T82.

The term "hydrophobic amino acid residue" comprises the amino acid residues V, L, I, M, F, W and Y.

The term "MIU" stands for Million International Unit and means the internationally established potency unit of measurement of interferon as defined by the International Conference for Unification of Formulae.

When used about polymer molecules herein, the word "about" indicates an approximate average molecular weight and reflects the fact that there will normally be a certain molecular weight distribution in a given polymer preparation.

A "patient" for the purposes of the present invention includes both humans and other mammals. Thus, the methods are applicable to both human therapy and veterinary applications.



**IFNB polypeptides for use in the invention**

Suitable IFNB polypeptides for use in the present invention are IFNB polypeptides with an increased functional *in vivo* half-life as compared to interferon  $\beta$ 1a. Such IFNB polypeptides may be achieved in numerous ways.

In one embodiment, said IFNB polypeptide is an IFNB variant comprising an amino acid sequence which differs from the amino acid sequence of wild-type human IFNB (SEQ ID NO 2) in 1-15 amino acid residues and wherein said variant comprises at least one introduced glycosylation site.

In another embodiment, said IFNB polypeptide is an IFNB variant comprising an amino acid sequence which differs from the amino acid sequence of wild-type human IFNB (SEQ ID NO 2) in 1-15 amino acid residues and wherein said variant comprises at least one introduced glycosylation site and at least one polymer molecule covalently attached to an amino acid residue of the IFNB polypeptide.

In yet another embodiment, said IFNB polypeptide is an IFNB variant comprising an amino acid sequence which differs from the amino acid sequence of wild-type human IFNB (SEQ ID NO 2) in 1-15 amino acid residues and wherein said variant comprises at least one polymer molecule covalently attached to an amino acid residue of the IFNB polypeptide.

In still another embodiment, said IFNB polypeptide has the amino acid sequence shown in SEQ ID: NO 2 and comprises at least one polymer molecule covalently attached to an amino acid residue of the IFNB polypeptide. Such conjugated polypeptides have been disclosed in WO 00/23114, where interferon  $\beta$ 1a has been PEGylated at the N-terminus or at the sugar moiety present at N80.

The biological activity of the IFNB polypeptides can be assayed by any suitable method known in the art. Such assays include tests for antiviral activity, induction of protein kinase, oligoadenylate 2,5-A synthetase or phosphodiesterase activities, as described in EP 0 41 313 B1. Such assays also include immunomodulatory assays (see, e.g., US 4,753,795), growth inhibition assays, and measurement of binding to cells that express interferon receptors.

Specific assays for determining the biological activity of polypeptides or conjugates for use in the invention are disclosed in the Materials and Methods section herein.

***IFNB polypeptides with introduced glycosylation sites***

In a preferred embodiment of the invention the IFNB polypeptide is a variant of wild-type human IFNB, wherein said variant comprises at least one introduced (additional) *in vivo*

glycosylation site. The introduced *in vivo* glycosylation site may be an O-glycosylation site, but is preferably an N-glycosylation site. In order to ensure that sugar moieties are covalently attached to the glycosylation sites it will be understood that such glycosylated variants must be produced in a host cell capable of glycosylation. Thus, according to this embodiment of the invention the IFNB polypeptide comprises at least one introduced glycosylation site, e.g. 1-6 or 1-4 introduced glycosylation sites (e.g. 1, 2, 3, 4, 5 or 6 introduced glycosylation sites). Accordingly, the IFNB polypeptide according to this embodiment of the invention comprises at least one introduced sugar moiety, such as 1-6 or 1-4 introduced sugar moieties (e.g. 1, 2, 3, 4, 5 or 6 introduced sugar moieties).

More particularly, the *in vivo* N-glycosylation site is introduced in a position of the parent IFNB molecule occupied by an amino acid residue exposed to the surface of the parent molecule, preferably with more than 25% of the side chain exposed to the surface, in particular with more than 50% exposed to the surface (these positions are identified in the Methods section herein). The *in vivo* N-glycosylation site is introduced in such a way that the N-residue of said site is located in said position. Analogously, an O-glycosylation site is introduced so that the S or T residue making up such site is located in said position. Furthermore, in order to ensure efficient glycosylation it is preferred that the *in vivo* glycosylation site, in particular the N residue of the N-glycosylation site or the S or T residue of the O-glycosylation site, is located within the first 141 amino acid residues of the IFNB polypeptide, more preferably within the first 116 amino acid residues.

Substitutions that lead to introduction of an *in vivo* N-glycosylation site at positions exposed at the surface of the parent IFNB molecule and occupied by amino acid residues having more than 25% of the side chain exposed to the surface, include substitutions selected from the group consisting of

S2N+N4S/T, L6S/T, L5N+G7S/T, F8N+Q10S/T, L9N+R11S/T, R11N, R11N+S13T, S12N+N14S/T, F15N+C17S/T, Q16N+Q18S/T, Q18N+L20S/T, K19N+L21S/T, W22N+L24S/T, Q23N+H25S/T, G26N+L28S/T, R27N+E29S/T, L28S+Y30S/T, Y30N+L32S/T, L32N+D34S/T, K33N+R35S/T, R35N+N37S/T, M36N+F38S/T, D39S/T, D39N+P41S/T, E42N+I44S/T, Q43N+K45S/T, K45N+L47S/T, Q46N+Q48S/T, L47N+Q49T/S, Q48N+F50S/T, Q49N+Q51S/T, Q51N+E53S/T, K52N+D54S/T, L57N+I59S/T, Q64N+I66S/T, A68N+F70S/T, R71N+D73S/T, Q72N, Q72N+S74T, D73N, D73N+S75T, S75N+T77S, S75N, S76N+G78S/T, E81N+I83S/T, T82N+V84S/T, E85N+L87S/T, L88S/T, A89N+V91S/T, Y92S/T, Y92N+Q94S/T, H93N+I95S/T, L98S/T,

H97N+K99S/T, K99N+V101S/T, T100N+L102S/T, E103N+K105S/T, E104N+L106S/T, K105N+E107S/T, E107N+E109S/T, K108N+D110S/T, E109N+F111S/T, D110N+T112S, D110N, F111N+R113S/T, R113N+K115S/T, G114N+L116S/T, K115N+M117S/T, L116N, L116N+S118T, S119N+H212S/T, L120N+L122S/T, H121N+K123S/T, K123N+Y125S/T, R124N+Y126S/T, G127N+I129S/T, R128N+L130S/T, L130N+Y132S/T, H131N+L133S/T, K134N+K136S/T, A135N+E137S/T, K136N+Y138S/T, E137N, Y138N+H140S/T, H140N+A142S/T, V148N+I150S/T, R152N+F154S/T, Y155N+I157S/T, L160S/T, R159N+T161S, R159N, G162N+L164S/T and Y163N+R165S/T.

Substitutions that lead to introduction of an *in vivo* N-glycosylation site at positions exposed at the surface of the parent IFNB molecule having more than 50% of the side chain exposed to the surface include substitutions selected from the group consisting of L6S/T, L5N+G7S/T, F8N+Q10S/T, L9N+R11S/T, S12N+N14S/T, F15N+C17S/T, Q16N+Q18S/T, K19N+L21S/T, W22N+L24S/T, Q23N+H25S/T, G26N+L28S/T, R27N+E29S/T, Y30N+L32S/T, K33N+R35S/T, R35N+N37S/T, M36N+F38S/T, D39S/T, D39N+P41S/T, E42N+I44S/T, Q46N+Q48S/T, Q48N+F50S/T, Q49N+Q51S/T, Q51N+E53S/T, K52N+D54S/T, L57N+I59S/T, R71N+D73S/T, D73N, D73N+S75T, S75N+T77S, S75N, S76N+G78S/T, E81N+I83S/T, T82N+V84S/T, E85N+L87S/T, A89N+V91S/T, Y92S/T, Y92N+Q94S/T, H93N+I95S/T, T100N+L102S/T, E103N+K105S/T, E104N+L106S/T, E107N+E109S/T, K108N+D110S/T, D110N+T112S, D110N, F111N+R113S/T, R113N+K115S/T, L116N, L116N+S118T, K123N+Y125S/T, R124N+Y126S/T, G127N+I129S/T, H131N+L133S/T, K134N+K136S/T, A135N+E137S/T, E137N, V148N+I150S/T and Y155N+I157S/T.

Among the substitutions mentioned in the above lists, those are preferred that have the N residue introduced among the 141 N-terminal amino acid residues, in particular among the 116 N-terminal amino acid residues.

The presently preferred substitutions include substitutions selected from the group consisting of S2N+N4T/S, L9N+R11T/S, R11N, S12N+N14T/S, F15N+C17S/T, Q16N+Q18T/S, K19N+L21T/S, Q23N+H25T/S, G26N+L28T/S, R27N+E29T/S, L28N+Y30T/S, D39T/S, K45N+L47T/S, Q46N+Q48T/S, Q48N+F50T/S, Q49N+Q51T/S, Q51N+E53T/S, R71N+D73T/S, Q72N, D73N, S75N, S76N+G78T/S, L88T/S, Y92T/S, N93N+I95T/S, L98T/S, E103N+K105T/S, E104N+L106T/S, E107N+E109T/S, K108N+D110T/S, D110N, F111N+R113T/S and L116N, more preferably selected from the group consisting of S2N+N4T, L9N+R11T, Q49N+Q51T, R71N+D73T and F111N+R113T,

even more preferably selected from the group consisting of Q49N+Q51T, R71N+D73T and F111N+R113T, most preferably selected from the group consisting of Q49N+Q51T and F111N+R113T.

The IFNB variant according to this embodiment of the invention may contain a single introduced *in vivo* N-glycosylation site. However, in order to obtain efficient shielding of epitopes present on the surface of the parent polypeptide and/or in order to obtain a sufficient increased *in vivo* functional half-life, it is often desirable that the polypeptide comprises at least two introduced glycosylation sites, such as 2-6 or 2-4 introduced glycosylation sites, such as 2, 3, 4, 5 or 6 introduced glycosylation sites. When the IFNB variant comprises two or more introduced glycosylation sites, the glycosylation sites are preferably introduced in the positions indicated above. Preferably, said glycosylation sites are *in vivo* N-glycosylation sites.

Specific examples of preferred IFNB variants comprising two or more introduced glycosylation sites may comprise substitutions selected from the group consisting of Q49N+Q51T+F111N+R113T, Q49N+Q51T+R71N+D73T+F111N+R113T, S2N+N4T+F111N+R113T, S2N+N4T+Q49N+Q51T, S2N+N4T+Q49N+Q51T+F111N+R113T, S2N+N4T+L9N+R11T+Q49N+Q51T, S2N+N4T+L9N+R11T+F111N+R113T, S2N+N4T+L9N+R11T+Q49N+Q51T+F111N+R113T, L9N+R11T+Q49N+Q51T, L9N+R11T+Q49N+Q51T+F111N+R113T and L9N+R11T+F111N+R113T.

Most preferably, the IFNB variant comprises the substitutions Q49N+Q51T+F111N+R113T (leading to introduction of two additional *in vivo* N-glycosylation sites).

It will be understood that in order to introduce a functional *in vivo* N-glycosylation site the amino acid residue in between the N-residue and the S/T residue is different from a proline residue. Normally, the amino acid residue "in between" will be that occupying the relevant position in the amino acid sequence shown in SEQ ID NO 2. For instance, in the polypeptide comprising the substitutions Q49N+Q51T, position 50 (occupied by a Phe residue) is the position "in between".

In a highly preferred embodiment, the IFNB polypeptide comprises three *in vivo* N-glycosylation sites (i.e. two additional (introduced) *in vivo* N-glycosylation sites (in addition to the naturally occurring N80 N-glycosylation site)), i.e. the IFNB variant comprises three *in vivo* N-glycosylation sites and three sugar moieties. In a particular preferred embodiment, the three *in vivo* N-glycosylation sites are located in positions 49, 80 and 111.

*Further amino acid modifications*

Any of the above-disclosed glycosylated variants may be further modified.

For example, it is presently preferred that the IFNB polypeptide is free from a cysteine residue. Thus, it is preferred that the cysteine residue located in position 17 of SEQ ID NO 2 has been removed, in particular by substitution, such as by the substitution C17S.

Specific examples of particular preferred IFNB variants, which comprises the C17S substitution, include variants comprising substitutions selected from the group consisting of S2N+N4T/S+C17S, L9N+R11T/S+C17S, R11N+C17S, S12N+N14T/S+C17S, F15N+C17S, Q16N+C17S+Q18T/S, C17S+K19N+L21T/S, C17S+Q23N+H25T/S, C17S+G26N+L28T/S, C17S+R27N+E29T/S, C17S+L28N+Y30T/S, C17S+D39T/S, C17S+K45N+L47T/S, C17S+Q46N+Q48T/S, C17S+Q48N+F50T/S, C17S+Q49N+Q51T/S, C17S+Q51N+E53T/S, C17S+R71N+D73T/S, C17S+Q72N, C17S+D73N, C17S+S75N, C17S+S76N+G78T/S, C17S+L88T/S, C17S+Y92T/S, C17S+N93N+I95T/S, C17S+L98T/S, C17S+E103N+K105T/S, C17S+E104N+L106T/S, C17S+E107N+E109T/S, C17S+K108N+D110T/S, C17S+D110N, C17S+F111N+R113T/S and C17S+L116N, more preferably selected from the group consisting of S2N+N4T+C17S, L9N+R11T+C17S, C17S+Q49N+Q51T, C17S+F111N+R113T, C17S+Q49N+Q51T+F111N+R113T, C17S+Q49N+Q51T+R71N+D73T+ F111N+R113T, S2N+N4T+C17S+F111N+R113T, S2N+N4T+C17S+Q49N+Q51T, S2N+N4T+C17S+Q49N+Q51T+F111N+R113T, S2N+N4T+L9N+R11T+C17S+Q49N+Q51T, S2N+N4T+L9N+R11T+C17S+F111N+R113T, S2N+N4T+L9N+R11T+C17S+Q49N+Q51T+F111N+R113T, L9N+R11T+C17S+Q49N+Q51T, L9N+R11T+C17S+Q49N+Q51T+F111N+R113T and L9N+R11T+C17S+F111N+R113T.

Most preferably, the IFNB variant comprises the substitutions C17S+Q49N+Q51T+F111N+R113T (leading to introduction of two additional *in vivo* N-glycosylation sites at positions 49 and 111, and removal of the cysteine residue at position 17).

In a further preferred embodiment, the IFNB variant further comprises one or more substitutions located close to a glycosylation site in order to optimize or increase the glycosylation at the site. Specific examples are described in the section entitled "*Variants with increased glycosylation*", pp. 14-23, in WO 02/074806.

In an interesting embodiment of the invention, the IFNB variant comprises an amino acid substitution in position 48, in particular if the variant comprises an introduced *in vivo* N-

glycosylation site in position 49. Preferably, the glutamine residue located at position 49 is substituted with a hydrophobic amino acid residue, such as Q48F, Q48V, Q48W or Q48Y.

In a highly preferred embodiment of the invention, the IFNB variant comprises an amino acid substitution in position 110, in particular if the variant comprises an introduced *in vivo* N-glycosylation site in position 111. Preferably, the aspartic acid residue located at position 110 is substituted with a hydrophobic amino acid residue, such as D110F, D110V, D110W or D110Y. In a particular preferred embodiment the variant comprises the substitution D110F, preferably in combination with the substitutions F111N+R113T/S, in particular F111N+R113T

Accordingly, specific examples of particular preferred IFNB variants include variants comprising substitutions selected from the group consisting of D110F+F111N+R113T, Q49N+Q51T+D110F+F111N+R113T, Q49N+Q51T+R71N+D73T+D110F+F111N+R113T, S2N+N4T+D110F+F111N+R113T, S2N+N4T+Q49N+Q51T+D110F+F111N+R113T, S2N+N4T+L9N+R11T+D110F+F111N+R113T, S2N+N4T+L9N+R11T+Q49N+Q51T+D110F+F111N+R113T, L9N+R11T+Q49N+Q51T+D110F+F111N+R113T and L9N+R11T+D110F+F111N+R113T, in particular Q49N+Q51T+D110F+F111N+R113T.

Even more preferably, the IFNB variant comprises substitutions selected from the group consisting of

C17S+D110F+F111N+R113T, C17S+Q49N+Q51T+D110F+F111N+R113T, C17S+Q49N+Q51T+R71N+D73T+D110F+F111N+R113T, S2N+N4T+C17S+D110F+F111N+R113T, S2N+N4T+C17S+Q49N+Q51T+D110F+F111N+R113T, S2N+N4T+L9N+R11T+C17S+D110F+F111N+R113T, S2N+N4T+L9N+R11T+C17S+Q49N+Q51T+D110F+F111N+R113T, L9N+R11T+C17S+Q49N+Q51T+D110F+F111N+R113T and L9N+R11T+C17S+D110F+F111N+R113T.

Most preferably, the IFNB variant comprises the substitutions C17S+Q49N+Q51T+D110F+F111N+R113T (SEQ ID NO 3):

#### *Polymer conjugation*

In a further embodiment of the invention the IFNB polypeptide comprises at least one polymer molecule covalently attached to an amino acid residue of the IFNB polypeptide.

As indicated above, the polymer molecule, such as PEG, including mPEG, is covalently attached to an amino acid residue of the IFNB polypeptide where the amino acid residue comprises an attachment group for the polymer molecule. Examples of such attachment groups are shown in the table on page 7-8 in WO 03/002152. Preferred attachment groups include the N-terminal amino group, the  $\epsilon$ -amino group of a lysine residue and the -S-H group of a cysteine residue, in particular the N-terminal amino group and the  $\epsilon$ -amino group of a lysine residue.

When the IFNB polypeptide is PEGylated it usually comprises 1-5 polyethylene glycol (PEG) molecules, such as 1-3 PEG molecules, e.g. 1, 2 or 3 PEG molecules. In a further embodiment each PEG molecule has a molecular weight of about 5 kDa (kilo Dalton) to 100 kDa, such as a molecular weight of about 10 kDa to 40 kDa, e.g. about 12 kDa or about 20 kDa.

In a particular preferred embodiment of the invention the IFNB polypeptide comprises a single PEG molecule, in particular a single PEG molecule having a molecular weight of about 20 kDa.

Suitable PEG molecules are available from Shearwater Polymers, Inc. and Enzon, Inc. and may be selected from SS-PEG, NPC-PEG, aldehyd-PEG, mPEG-SPA, mPEG-SCM, mPEG-BTC, SC-PEG, tresylated mPEG (US 5,880,255), or oxycarbonyl-oxy-N-dicarboxyimide-PEG (US 5,122,614).

In one embodiment of this aspect of the invention the IFNB polypeptide has the amino acid sequence shown in SEQ ID NO 2. In another embodiment of this aspect of the invention the IFNB polypeptide comprises the substitution C17S. In any of these situations the PEG polymer may be selectively conjugated to either the N-terminus or the naturally occurring sugar moiety present in position 80, such as described in WO 00/23114.

In still another embodiment of the invention at least one lysine residue has been removed. For example, said lysine residue may be selected from the group consisting of K19, K33, K45, K52, K99, K105, K108, K115, K123, K134 and K136, preferably from the group consisting of K19, K33, K45 and K123. The lysine residue to be removed is preferably substituted with an arginine or glutamine residue, in particular with an argine residue. Specific examples of such substitutions may be selected from the group consisting of K19R, K33R, K45R, K123R, K19R+K33R, K19R+K45R, K19R+K123R, K33R+K45R, K33R+K123R, K45R+K123R, K19R+K45R+K123R, K19R+K33R+K123R, K19R+K33R+K45R, K33R+K45R+K123R and K19R+K33R+K45R+K123R, more preferably selected from the

group consisting of K19R+K45R+K123R, K19R+K33R+K123R, K19R+K33R+K45R and K33R+K45R+K123R, most preferably K19R+K33R+K45R.

In a highly preferred embodiment of this aspect of the invention the IFNB polypeptide is a variant of human wild-type IFNB, i.e. said IFNB polypeptide is an IFNB variant comprising an amino acid sequence which differs from the amino acid sequence of wild-type human IFNB (SEQ ID NO 2) in 1-15 amino acid residues and wherein said variant comprises at least one polymer molecule covalently attached to an amino acid residue of the IFNB polypeptide. In one embodiment according to this aspect of the invention no glycosylation sites have been introduced. However, in a particular preferred embodiment of this aspect of the invention, the IFNB polypeptide is an IFNB variant comprising an amino acid sequence which differs from the amino acid sequence of wild-type human IFNB (SEQ ID NO 2) in 1-15 amino acid residues and wherein said variant comprises at least one polymer molecule covalently attached to an amino acid residue of the IFNB polypeptide and wherein said variant comprises at least one introduced *in vivo* N-glycosylation site. As will be understood the *in vivo* N-glycosylation site may be introduced in any of the position indicated in the section entitled "*IFNB polypeptides with introduced glycosylation sites*". More preferably, these glycosylated and conjugated IFNB variants further comprises a modification as disclosed in the section entitled "*Further amino acid modifications*"

Accordingly, a particular preferred IFNB variant according to this embodiment of the invention is an IFNB variant comprising an amino acid sequence which differs from the amino acid sequence of wild-type human IFNB (SEQ ID NO 2) in 1-15 amino acid residues and wherein said variant comprises at least one polymer molecule covalently attached to an amino acid residue of the IFNB polypeptide and wherein said variant comprises at least one introduced *in vivo* N-glycosylation site selected from the group consisting of S2N+N4T/S+C17S, L9N+R11T/S+C17S, R11N+C17S, S12N+N14T/S+C17S, F15N+C17S, Q16N+C17S+Q18T/S, C17S+K19N+L21T/S, C17S+Q23N+H25T/S, C17S+G26N+L28T/S, C17S+R27N+E29T/S, C17S+L28N+Y30T/S, C17S+D39T/S, C17S+K45N+L47T/S, C17S+Q46N+Q48T/S, C17S+Q48N+F50T/S, C17S+Q49N+Q51T/S, C17S+Q51N+E53T/S, C17S+R71N+D73T/S, C17S+Q72N, C17S+D73N, C17S+S75N, C17S+S76N+G78T/S, C17S+L88T/S, C17S+Y92T/S, C17S+N93N+I95T/S, C17S+L98T/S, C17S+E103N+K105T/S, C17S+E104N+L106T/S, C17S+E107N+E109T/S, C17S+K108N+D110T/S, C17S+D110N, C17S+F111N+R113T/S and C17S+L116N, more preferably selected from the group consisting of S2N+N4T+C17S, L9N+R11T+C17S, C17S+Q49N+Q51T, C17S+F111N+R113T,



C17S+Q49N+Q51T+F111N+R113T, C17S+Q49N+Q51T+R71N+D73T+ F111N+R113T,  
 S2N+N4T+C17S+F111N+R113T, S2N+N4T+C17S+Q49N+Q51T,  
 S2N+N4T+C17S+Q49N+Q51T+F111N+R113T, S2N+N4T+L9N+R11T+C17S+Q49N+Q51T,  
 S2N+N4T+L9N+R11T+C17S+F111N+R113T,  
 S2N+N4T+L9N+R11T+C17S+Q49N+Q51T+F111N+R113T,  
 L9N+R11T+C17S+Q49N+Q51T, L9N+R11T+C17S+Q49N+Q51T+F111N+R113T and  
 L9N+R11T+C17S+F111N+R113T

Most preferably, the IFNB variant comprises the substitutions C17S+Q49N+Q51T+  
 F111N+R113.

In a particular preferred embodiment of the invention said conjugated and glycosylated  
 IFNB variant further comprises the substitution C17S and/or D110F/V/W/Y, in particular  
 C17S and D110F. Specific examples include conjugated variants comprising substitutions  
 selected from the group consisting of

C17S+D110F+F111N+R113T,  
 C17S+Q49N+Q51T+D110F+F111N+R113T,  
 C17S+Q49N+Q51T+R71N+D73T+D110F+F111N+R113T,  
 S2N+N4T+C17S+D110F+F111N+R113T,  
 S2N+N4T+C17S+Q49N+Q51T+D110F+F111N+R113T,  
 S2N+N4T+L9N+R11T+C17S+D110F+F111N+R113T,  
 S2N+N4T+L9N+R11T+C17S+Q49N+Q51T+D110F+F111N+R113T,  
 L9N+R11T+C17S+Q49N+Q51T+D110F+F111N+R113T and  
 L9N+R11T+C17S+D110F+F111N+R113T.

Most preferably, the conjugated IFNB variant comprises the substitutions  
 C17S+Q49N+Q51T+ D110F+F111N+R113T (SEQ ID NO 3).

In a further embodiment at least one lysine residue has been removed. For example, said  
 lysine residue may be selected from the group consisting of K19, K33, K45, K52, K99, K105,  
 K108, K115, K123, K134 and K136, preferably from the group consisting of K19, K33, K45  
 and K123. The lysine residue to be removed is preferably substituted with an arginine or  
 glutamine residue, in particular with an arginine residue. Specific examples of such substitutions  
 may be selected from the group consisting of K19R, K33R, K45R, K123R, K19R+K33R,  
 K19R+K45R, K19R+K123R, K33R+K45R, K33R+K123R, K45R+K123R,  
 K19R+K45R+K123R, K19R+K33R+K123R, K19R+K33R+K45R, K33R+K45R+K123R and  
 K19R+K33R+K45R+K123R, more preferably selected from the group consisting of

K19R+K45R+K123R, K19R+K33R+K123R, K19R+K33R+K45R and K33R+K45R+K123R, most preferably K19R+K33R+K45R.

Specific examples include conjugated variants comprising substitutions selected from the group consisting of

C17S+Q49N+Q51T+K19R+K33R+K45R,

C17S+D110F+F111N+R113T+K19R+K33R+K45R,

C17S+Q49N+Q51T+D110F+F111N+R113T+K19R+K33R+K45R,

C17S+Q49N+Q51T+R71N+D73T+D110F+F111N+ R113T+K19R+K33R+K45R,

S2N+N4T+C17S+D110F+F111N+R113T+K19R+K33R+K45R,

S2N+N4T+C17S+Q49N+Q51T+D110F+F111N+R113T+K19R+K33R+K45R,

S2N+N4T+L9N+R11T+C17S+D110F+F111N+R113T+K19R+K33R+K45R,

S2N+N4T+L9N+R11T+C17S+Q49N+Q51T+D110F+F111N+R113T+K19R+K33R+K45R,

L9N+R11T+C17S+Q49N+Q51T+D110F+F111N+R113T+K19R+K33R+K45R and

L9N+R11T+C17S+D110F+F111N+R113T+K19R+K33R+K45R.

Most preferably, the conjugated and glycosylated IFNB variant comprises the substitutions C17S+Q49N+Q51T+D110F+F111N+R113T+K19R+K33R+K45R (SEQ ID NO 4).

## Methods of preparation

### *Methods of preparing a conjugate*

Specific details concerning conjugation of polymer molecules, in particular PEG polymers, to the IFNB variants disclosed herein are given in the section entitled "*Methods of preparing a conjugate of the invention*", pp. 32-40, in WO 01/15736

### *Coupling to a sugar moiety*

In order to achieve *in vivo* glycosylation of an IFNB polypeptide as described herein, the nucleotide sequence encoding the IFNB variant must be inserted in a glycosylating, eucaryotic expression host, such as an CHO cell. Suitable expression host cells are described in the section entitled "*coupling to a sugar moiety*", p. 36, in WO 01/15736.

*Methods of preparing an IFNB polypeptide variant*

The IFNB variants for use in the present invention may be produced by any suitable method known in the art. Such methods include constructing a nucleotide sequence encoding the polypeptide variant and expressing the sequence in a suitable transformed or transfected host. However, polypeptides for use in the invention may be produced, albeit less efficiently, by chemical synthesis or a combination of chemical synthesis or a combination of chemical synthesis and recombinant DNA technology.

The nucleotide sequence encoding an IFNB polypeptide for use in the present invention may be constructed by isolating or synthesizing a nucleotide sequence encoding the parent IFNB, e.g. with the amino acid sequence shown in SEQ ID NO 2, and then changing the nucleotide sequence so as to effect introduction (i.e. insertion or substitution) or removal (i.e. deletion or substitution) of the relevant amino acid residue(s).

The nucleotide sequence is conveniently modified by site-directed mutagenesis in accordance with well-known methods, see, e.g., Mark et al., "Site-specific Mutagenesis of the Human Fibroblast Interferon Gene", Proc. Natl. Acad. Sci. USA, 81, pp. 5662-66 (1984); and US 4,588,585.

Alternatively, the nucleotide sequence is prepared by chemical synthesis, e.g. by using an oligonucleotide synthesizer, wherein oligonucleotides are designed based on the amino acid sequence of the desired polypeptide, and preferably selecting those codons that are favoured in the host cell in which the recombinant polypeptide will be produced. For example, several small oligonucleotides coding for portions of the desired polypeptide may be synthesized and assembled by PCR, ligation or ligation chain reaction (LCR). The individual oligonucleotides typically contain 5' or 3' overhangs for complementary assembly.

Once assembled (by synthesis, site-directed mutagenesis or another method), the nucleotide sequence encoding the IFNB polypeptide is inserted into a recombinant vector and operably linked to control sequences necessary for expression of the IFNB variant in the desired transformed host cell.

A detailed description of the production of the IFNB variants disclosed herein, including suitable expression vectors, control sequences, host cells, production media, purification techniques, etc. can be found in the section entitled "*Methods of preparing an interferon  $\beta$  polypeptide for use in the invention*", pp. 43-51, in WO 01/15736

### Pharmaceutical composition

In a further aspect the invention relates to a pharmaceutical composition comprising

- i) an interferon beta (IFNB) polypeptide with increased functional *in vivo* half-life as compared to interferon  $\beta$ 1a;
- ii) a chemotherapeutic agent; and
- iii) a pharmaceutically acceptable diluent, carrier or adjuvant.

In a highly preferred embodiment of the invention, the IFNB polypeptide has the amino acid sequence shown in SEQ ID NO 3 or 4 and is conjugated to a polymer molecule, such as a PEG molecule, more preferably a PEG molecule having a molecular weight of about 12 kDa or about 20 kDa, in particular about 20 kDa. Most preferably, only one PEG group is covalently attached to the IFNB polypeptide. The PEG polymer is preferably covalently attached to the  $\epsilon$ -amino group of a lysine residue or the N-terminal amino group.

The chemotherapeutic agent may be selected from the group consisting of adrenocorticosteroids, such as prednisone, dexamethasone or decadron; altretamine (hexalen, hexamethylmelamine (HMM)); amifostine (ethyol); aminoglutethimide (cytadren); amsacrine (M-AMSA); anastrozole (arimidex); androgens, such as testosterone; asparaginase (elspar); bacillus calmette-gurin; bicalutamide (casodex); bleomycin (blenoxane); busulfan (myleran); carboplatin (paraplatin); carmustine (BCNU, BiCNU); chlorambucil (leukeran); chlorodeoxyadenosine (2-CDA, cladribine, leustatin); cisplatin (platinol); cytosine arabinoside (cytarabine); dacarbazine (DTIC); dactinomycin (actinomycin-D, cosmegen); daunorubicin (cerubidine); docetaxel (taxotere); doxorubicin (adriamycin); epirubicin; estramustine (emcyt); estrogens, such as diethylstilbestrol (DES); etoposide (VP-16, VePesid, etopophos); fludarabine (fludara); flutamide (eulexin); 5-FUDR (floxuridine); 5-fluorouracil (5-FU); gemcitabine (gemzar); goserelin (zodalex); herceptin (trastuzumab); hydroxyurea (hydrea); idarubicin (idamycin); ifosfamide; IL-2 (proleukin, aldesleukin); interferon alpha (intron A, roferon A); irinotecan (camptosar); leuprolide (lupron); levamisole (ergamisole); lomustine (CCNU); mechlorathamine (mustargen, nitrogen mustard); melphalan (alkeran); mercaptopurine (purinethol, 6-MP); methotrexate (mexate); mitomycin-C (mutamucin); mitoxantrone (novantrone); octreotide (sandostatin); pentostatin (2-deoxycoformycin, nipent); plicamycin (mithramycin, mithracin); prorocarbazine (matulane); streptozocin; tamoxifen (nolvadex); taxol (paclitaxel); teniposide (vumon, VM-26); thiotepa; topotecan (hycamtin); tretinoin (vesanoid, all-trans retinoic acid); vinblastine (valban); vincristine (oncovin) and vinorelbine (navelbine).

More preferably, the chemotherapeutic agent is selected from the group consisting of cisplatin, IL-2, doxorubicin, taxol and 5-fluorouracil. It will be understood, however, that the chemotherapeutic agent is selected under due consideration of the actual cancer form to be treated.

The IFNB polypeptides can be used "as is" and/or in a salt form thereof. Suitable salts include, but are not limited to, salts with alkali metals or alkaline earth metals, such as sodium, potassium, lithium, calcium and magnesium, as well as e.g. zinc salts. These salts or complexes may be present as a crystalline and/or amorphous structure.

The IFNB polypeptide is preferably administered in a composition further including a pharmaceutically acceptable carrier or excipient. "Pharmaceutically acceptable" means a carrier or excipient that does not cause any untoward effects in patients to whom it is administered. Such pharmaceutically acceptable carriers and excipients are well known in the art.

The IFNB polypeptide can be formulated into pharmaceutical compositions by well-known methods. Suitable formulations are described in US 5,183,746, Remington's Pharmaceutical Sciences by E.W. Martin, 18<sup>th</sup> edition, A. R. Gennaro, Ed., Mack Publishing Company [1990]; Pharmaceutical Formulation Development of Peptides and Proteins, S. Frokjaer and L. Hovgaard, Eds., Taylor & Francis [2000]; and Handbook of Pharmaceutical Excipients, 3rd edition, A. Kibbe, Ed., Pharmaceutical Press [2000]).

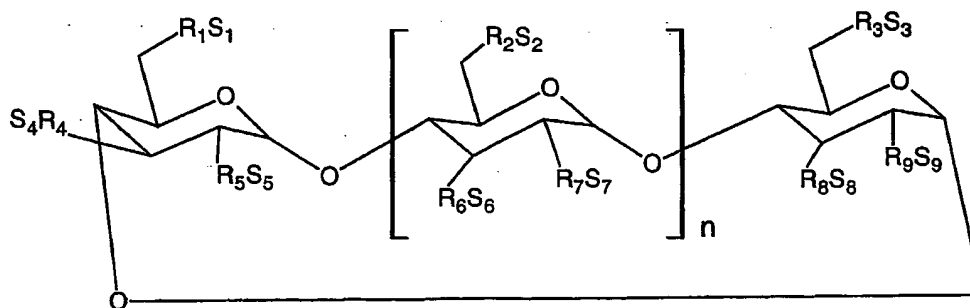
The IFNB polypeptide may be formulated into a pharmaceutical composition in a variety of forms, including liquid, gel, lyophilized, pulmonary dispersion, or any other suitable form, e.g. as a compressed solid. The preferred form will depend upon the particular indication being treated and will be apparent to one of skill in the art.

The pharmaceutical composition may be administered parenterally (e.g. intravenously, intramuscularly, intraperitoneally, or subcutaneously), orally, intracerebrally, intradermally, intranasally, intrapulmonary, by inhalation, or in any other acceptable manner, e.g. using PowderJect or ProLease technology. The preferred mode of administration will depend upon the particular indication being treated and will be apparent to one of skill in the art.

A detailed description of suitable pharmaceutical compositions is given in the section entitled "*Pharmaceutical composition and uses of a conjugate of the invention*", pp. 52-61 in WO 01/15736.

In a preferred embodiment of the invention the pharmaceutical composition is a liquid composition and comprises a sulfoalkyl ether cyclodextrin derivative of the formula

29



wherein

$n$  is 4, 5 or 6;  $R_1$ ,  $R_2$ ,  $R_3$ ,  $R_4$ ,  $R_5$ ,  $R_6$ ,  $R_7$ ,  $R_8$ , and  $R_9$  are each, independently, -O- or a -O-(C<sub>2</sub>-C<sub>6</sub> alkyl)-SO<sub>3</sub><sup>-</sup> group, wherein at least one of  $R_1$ ,  $R_2$  or  $R_3$  is independently a -O-(C<sub>2</sub>-C<sub>6</sub> alkyl)-SO<sub>3</sub><sup>-</sup> group; and  $S_1$ ,  $S_2$ ,  $S_3$ ,  $S_4$ ,  $S_5$ ,  $S_6$ ,  $S_7$ ,  $S_8$ , and  $S_9$  are each, independently, a pharmaceutically acceptable cation, including H<sup>+</sup>.

It should be noted that when  $n=4$ , the sulfoalkyl ether cyclodextrin may also be referred to as a  $\alpha$ -sulfoalkyl ether cyclodextrin. In a similar way, when  $n=5$ , the term  $\beta$ -sulfoalkyl ether cyclodextrin may be employed and when  $n=6$ , the sulfoalkyl ether cyclodextrin may also be referred to as a  $\gamma$ -sulfoalkyl ether cyclodextrin.

In a further embodiment,  $n$  is 5 or 6. In a preferred embodiment  $n=6$ .

In a still further embodiment  $R_1$ ,  $R_2$  or  $R_3$  is independently selected from the group consisting of -OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>SO<sub>3</sub><sup>-</sup>, -OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>SO<sub>3</sub><sup>-</sup> and -OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>SO<sub>3</sub><sup>-</sup>. Most preferably,  $R_1$ ,  $R_2$  or  $R_3$  is independently -OCH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>CH<sub>2</sub>SO<sub>3</sub><sup>-</sup>.

In a further embodiment  $S_1$ ,  $S_2$ ,  $S_3$ ,  $S_4$ ,  $S_5$ ,  $S_6$ ,  $S_7$ ,  $S_8$ , and  $S_9$  are each, independently, a pharmaceutically acceptable cation selected from H<sup>+</sup>, alkali metals (e.g. Li<sup>+</sup>, Na<sup>+</sup>, K<sup>+</sup>), alkaline earth metals (e.g., Ca<sup>+2</sup>, Mg<sup>+2</sup>), ammonium ions and amine cations such as the cations of (C<sub>1</sub>-C<sub>6</sub>) alkylamines, piperidine, pyrazine, (C<sub>1</sub>-C<sub>6</sub>) alkanolamine and (C<sub>4</sub>-C<sub>8</sub>) cycloalkanolamine. Most preferably,  $S_1$ ,  $S_2$ ,  $S_3$ ,  $S_4$ ,  $S_5$ ,  $S_6$ ,  $S_7$ ,  $S_8$ , and  $S_9$  are each, independently, a pharmaceutically acceptable cation selected from the group consisting of H<sup>+</sup>, Li<sup>+</sup>, Na<sup>+</sup>, K<sup>+</sup>, in particular Na<sup>+</sup>.

The sulfoalkyl ether cyclodextrin may contain from 1 to 18 sulfoalkyl groups (when  $n=4$ ), from 1-21 sulfoalkyl groups (when  $n=5$ ) or from 1-21 (when  $n=6$ ). In a preferred embodiment of the invention  $n=5$  and the sulfoalkylether derivative comprises, on average, 2-20 sulfoalkyl groups (in particular sulfobutyl groups), such as 3-10 sulfoalkyl groups (in particular sulfobutyl groups), more preferably 4-9 sulfoalkyl groups (in particular sulfobutyl groups), even more preferably 5-9 sulfoalkyl groups (in particular sulfobutyl groups), such as 6-

8 sulfoalkyl groups (in particular sulfobutyl groups), e.g. 7 sulfoalkyl groups (in particular sulfobutyl groups).

The presently preferred sulfoalkyl ether cyclodextrin derivative is a salt, in particular the sodium salt, of  $\beta$ -cyclodextrin sulfobutyl ether (i.e.  $n=5$ ), which on average contains 7 sulfobutyl groups. This sulfoalkyl ether cyclodextrin derivative is also termed SBE7- $\beta$ -CD and is available as Captisol® (Cydex, Overland Park, Kansas 66213, US).

Further details concerning pharmaceutical compositions comprising the IFNB polypeptides disclosed herein and sulfoalkyl ether cyclodextrin derivatives can be found in the section entitled "*The sulfoalkyl ether cyclodextrin derivative*", pp. 37-49, in WO 03/002152.

### Use in cancer therapies

The present invention provides means and methods for treating certain cancers or tumors or tumour angiogenesis, in any suitable animal, preferably mammal, and in particular human.

Thus, the main aspect of the present invention relates to the use of an interferon  $\beta$  (IFNB) polypeptide with an increased functional *in vivo* half-life as compared to interferon  $\beta$ 1a for the the manufacture of a medicament for the treatment of a cancer having malignant cells carrying interferon type 1 deletions, in particular where said malignant cells have a deletion of the short arm of chromosome 9 (9p22).

Analogously, the present invention also relates to a method of treating a mammal, in particular a human being, having a cancer, wherein said cancer has malignant cells carrying interferon type 1 deletions, said method comprising administering to said mammal a therapeutically effective dose of an interferon beta (IFNB) polypeptide with increased functional *in vivo* half-life as compared to interferon  $\beta$ 1a.

In the present context the term "treatment of a cancer" or treating a mammal having a cancer" is intended to mean that the IFNB polypeptides described herein are administered in a therapeutically effective amount which is sufficient to *i*) inhibit growth of the tumor, *ii*) facilitate tumor regression, i.e. reduce the size of the tumor, *iii*) remove the tumor and/or *iv*) inhibit cancer cell metastasis.

The IFNB polypeptides described herein will be administered to patients in a "therapeutically effective" dose, i.e. a dose that is sufficient to produce the desired effects in relation to the condition for which it is administered. The exact dose will depend on the cancer form to be treated, and will be ascertainable by one skilled in the art using known techniques. A

suitable dose of an IFNB polypeptide described herein is contemplated to be in the range of about 1-500 microgram/kg body weight (based on the weight of the protein part of the conjugate), e.g. 2-200 microgram/kg, such as 3-100 microgram/kg or 5-50 microgram/kg. In previous clinical trials, with IFNB non-small cell lung cancer patients were treated with a weekly dose of 17 microgram/kg/week (Byhardt et al *J. Interferon Cytokine Res.* 16:891, 1996). It should be noted that a similar dose (in units) of pegylated IFNB with a specific activity four times lower than non-pegylated IFNB would correspond to 49 microgram/kg/week

In a very interesting embodiment of the invention said cancer is a carcinoma, such as a carcinoma selected from the group consisting of malignant melanoma, basal cell carcinoma, ovarian carcinoma, breast carcinoma, non-small cell lung cancer, renal cell carcinoma, bladder carcinoma, recurrent superficial bladder cancer, stomach carcinoma, prostatic carcinoma, pancreatic carcinoma, lung carcinoma, cervical carcinoma, cervical dysplasia, laryngeal papillomatosis, colon carcinoma, colorectal carcinoma and carcinoid tumors, in particular selected from the group consisting of malignant melanoma, non-small cell lung cancer, breast carcinoma, colon carcinoma and renal cell carcinoma.

Thus, in one embodiment of the invention said cancer is malignant melanoma, such as superficial spreading melanoma, nodular melanoma, lentigo maligna melanoma, acral melanoma, amelanotic melanoma or desmoplastic melanoma. In another embodiment of the invention said cancer is non-small cell lung cancer. In still another embodiment of the invention said cancer is breast carcinoma. In a further embodiment of the invention said cancer is colon carcinoma. In an even further embodiment of the invention said cancer is renal cell carcinoma.

In a further interesting embodiment of the invention said cancer is a sarcoma, such as a sarcoma selected from the group consisting of osteosarcoma, Ewing's sarcoma, chondrosarcoma, malignant fibrous histiocytoma, fibrosarcoma and Kaposi's sarcoma.

In an even further interesting embodiment of the invention said cancer is a glioma.

As will be understood by the skilled person, the above-mentioned cancer types (i.e. carcinomas, sarcomas and gliomas) are characterized by the presence of solid tumors. In these cases the IFNB polypeptide may be administered intratumorally, e.g. by direct injection into the tumor. In another alternative embodiment, the IFNB polypeptide may be administered intratumorally by gene therapy. As far as gene therapy is concerned one may use a nucleotide sequence encoding an IFNB polypeptide as described herein. In particular, it may be of interest to use a nucleotide sequence encoding an IFNB polypeptide as described in the sections above entitled "*IFNB polypeptides with introduced glycosylation sites*" and/or "*Further amino acid*



*modifications*". The glycosylation of the IFNB polypeptides is thus achieved during the course of the gene therapy, i.e. after expression of the nucleotide sequence in the human body.

Gene therapy applications contemplated include treatment of those diseases in which IFNB is expected to provide an effective therapy due to its antiproliferative activity, i.e., tumors and cancers, or other conditions characterized by undesired cell proliferation. A further description of such gene therapy is provided in WO 95/25170.

Local delivery of IFNB using gene therapy may provide the therapeutic agent to the target area while avoiding potential toxicity problems associated with non-specific administration. Both *in vitro* and *in vivo* gene therapy methodologies are contemplated.

Several methods for transferring potentially therapeutic genes to defined cell populations are known. For further reference see, e.g., Mulligan, "The Basic Science Of Gene Therapy", *Science*, 260, pp. 926-31 (1993). These methods include:

i) Direct gene transfer, e.g., as disclosed by Wolff et al., "Direct Gene transfer Into Mouse Muscle In vivo", *Science* 247, pp. 1465-68 (1990);

ii) Liposome-mediated DNA transfer, e.g., as disclosed by Caplen et al., "Liposome-mediated CFTR Gene Transfer to the Nasal Epithelium Of Patients With Cystic Fibrosis" *Nature Med.*, 3, pp. 39-46 (1995); Crystal, "The Gene As A Drug", *Nature Med.*, 1, pp.15-17 (1995); Gao and Huang, "A Novel Cationic Liposome Reagent For Efficient Transfection of Mammalian Cells", *Biochem.Biophys Res. Comm.*, 179, pp. 280-85 (1991);

iii) Retrovirus-mediated DNA transfer, e.g., as disclosed by Kay et al., "In vivo Gene Therapy of Hemophilia B: Sustained Partial Correction In Factor IX-Deficient Dogs", *Science*, 262, pp. 117-19 (1993); Anderson, "Human Gene Therapy", *Science*, 256, pp.808-13(1992);

iv) DNA Virus-mediated DNA transfer. Such DNA viruses include adenoviruses (preferably Ad-2 or Ad-5 based vectors), herpes viruses (preferably herpes simplex virus based vectors), and parvoviruses (preferably "defective" or non-autonomous parvovirus based vectors, more preferably adeno-associated virus based vectors, most preferably AAV-2 based vectors). See, e.g., Ali et al., "The Use Of DNA Viruses as Vectors for Gene Therapy", *Gene Therapy*, 1, pp. 367-84 (1994); US 4,797,368, and US 5,139,941.

v) Encapsulation therapy using genetically engineered cell lines. "In vitro study of encapsulation therapy for Fabry disease using genetically engineered CHO cell line." By Naganawa Y et al., *Cell Transplant.* 2002;11(4):325-9.

vi) Grafting of genetically modified cells that express therapeutic products. "Grafting of encapsulated BDNF-producing fibroblasts into the injured spinal cord without immune suppression in adult rats." By Tobias CA et al., *J Neurotrauma* 2001 Mar;18(3):287-301.

vii) Microencapsulation, as a tool for immunoisolation for allogenic or xenogenic implants. "Development of cellulose sulfate-based polyelectrolyte complex microcapsules for medical applications." By Dautzenberg H et al., *Ann N Y Acad Sci.* 1999 Jun 18;875:46-63.

One of the main purposes of treating a solid malignant tumor is to reduce the tumor so that it can be removed by surgery. Evidently, it would be desirable to completely remove the tumor by therapeutic treatment. Since a clear dose-dependent effect was seen with the tested IFNB polypeptides, a significant reduction in size, or even complete removal, of the solid malignant solid tumor should be possible.

Accordingly, in a further embodiment of the invention the IFNB polypeptides described herein have an increased growth inhibitory effect on the solid malignant tumor as compared to interferon  $\beta$ 1a (e.g. Rebif®) when tested under comparable conditions. Preferably, the solid malignant tumor shows no re-growth after treatment with the IFNB polypeptides described herein. In a further embodiment the solid malignant tumor is completely removed after treatment with the IFNB polypeptides described herein.

In a still further interesting embodiment of the invention said cancer is a leukaemia, such as a leukaemia selected from the group consisting of acute myelogenous leukaemia, chronic myelogenous leukaemia, acute lymphocytic leukaemia, chronic lymphocytic leukaemia and hairy cell leukaemia.

In an even further interesting embodiment of the invention said cancer is a lymphoma, such as a lymphoma selected from the group consisting of Hodgkin's disease, non-Hodgkin's disease, B-cell lymphoma, T-cell lymphoma, follicular lymphoma, Burkitt's lymphoma and mycosis fungoides.

In still another interesting embodiment of the invention said cancer is a myeloma, such as multiple myeloma.

It will be understood that an even more effective treatment of the various cancer forms may be obtained by combination therapy where an IFNB polypeptide as disclosed herein is combined with a suitable chemotherapeutic agent and/or is combined with radiotherapy.

Thus, a further aspect the present invention relates to the use of an interferon  $\beta$  (IFNB) polypeptide with an increased functional *in vivo* half-life as compared to interferon  $\beta$ 1a in

combination with a chemotherapeutic agent for the manufacture of a medicament for the treatment of cancer, such as any of the cancer forms mentioned above.

Specific examples of suitable chemotherapeutic agents include chemotherapeutic agents selected from the group consisting of adrenocorticosteroids, such as prednisone, dexamethasone or decadron; altretamine (hexalen, hexamethylmelamine (HMM)); amifostine (ethyol); aminoglutethimide (cytadren); amsacrine (M-AMSA); anastrozole (arimidex); androgens, such as testosterone; asparaginase (elspar); bacillus calmette-gurin; bicalutamide (casodex); bleomycin (blenoxane); busulfan (myleran); carboplatin (paraplatin); carmustine (BCNU, BiCNU); chlorambucil (leukeran); chlorodeoxyadenosine (2-CDA, cladribine, leustatin); cisplatin (platinol); cytosine arabinoside (cytarabine); dacarbazine (DTIC); dactinomycin (actinomycin-D, cosmegen); daunorubicin (cerubidine); docetaxel (taxotere); doxorubicin (adriamycin); epirubicin; estramustine (emcyt); estrogens, such as diethylstilbestrol (DES); etoposide (VP-16, VePesid, etopophos); fludarabine (fludara); flutamide (eulexin); 5-FUDR (floxuridine); 5-fluorouracil (5-FU); gemcitabine (gemzar); goserelin (zodalex); herceptin (trastuzumab); hydroxyurea (hydrea); idarubicin (idamycin); ifosfamide; IL-2 (proleukin, aldesleukin); interferon alpha (intron A, roferon A); irinotecan (camptosar); leuprolide (lupron); levamisole (ergamisole); lomustine (CCNU); mechlorathamine (mustargen, nitrogen mustard); melphalan (alkeran); mercaptopurine (purinethol, 6-MP); methotrexate (mexate); mitomycin-C (mutamucin); mitoxantrone (novantrone); octreotide (sandostatin); pentostatin (2-deoxycoformycin, nipent); plicamycin (mithramycin, mithracin); prorocarbazine (matulane); streptozocin; tamoxifen (nolvadex); taxol (paclitaxel); teniposide (vumon, VM-26); thiotepa; topotecan (hycamtin); tretinoin (vesanoid, all-trans retinoic acid); vinblastine (valban); vincristine (oncovin) and vinorelbine (navelbine).

Other chemotherapeutic agents, which may be useful for the purposes described herein include the chemotherapeutic agents mentioned in column 12, line 62 to column 13, line 42 of US 6,482,802. For a description of these and other chemotherapeutic agents, see *The Merck Index*, 12<sup>th</sup> edition, pp. THER 13-14.

It will be understood that the chemotherapeutic agent is selected under due consideration of the actual cancer form. In a preferred embodiment of the invention the following chemotherapeutic agents are used (together with the IFNB polypeptides described herein) for treatment of the following specific cancer forms: malignant melanoma and cisplatin; malignant melanoma and IL-2; renal cell carcinoma and doxorubicin; renal cell carcinoma and

IL-2; breast carcinoma and doxorubicin; breast carcinoma and taxol; colon carcinoma and 5-fluorouracil; colon carcinoma and cisplatin; and non-small cell lung cancer and cisplatin.

Moreover, in a further aspect the present invention relates to the use of an interferon  $\beta$  (IFNB) polypeptide with an increased functional *in vivo* half-life as compared to interferon  $\beta$ 1a for the the manufacture of a medicament for the treatment of a mammal having a cancer, where said mammal has circulating antibodies against interferon  $\beta$ 1a or interferon  $\beta$ 1b. As will be understood, any of the cancer forms discussed above may be subject for the treatment.

Analogously, the present invention also relates to a method of treating a mammal, in particular a human being, having a cancer, where said mammal has circulating antibodies against interferon  $\beta$ 1a or interferon  $\beta$ 1b, said method comprising administering to said mammal a therapeutically effective dose of an interferon beta (IFNB) polypeptide with increased functional *in vivo* half-life as compared to interferon  $\beta$ 1a.

In general, it is preferred that the IFNB polypeptide to be used in any of the methods described in this section has the amino acid sequence shown in SEQ ID NO 3 or 4 and is conjugated to a polymer molecule, such as a PEG molecule, more preferably a PEG molecule having a molecular weight of about 12 kDa or about 20 kDa, in particular about 20 kDa. Most preferably, only one PEG group is covalently attached to the IFNB polypeptide. The PEG polymer is preferably covalently attached to the  $\epsilon$ -amino group of a lysine residue or the N-terminal amino group.

Dependent on the cancer form to be treated, the efficiency of the IFNB polypeptides disclosed herein may be initially assessed in a suitable model. For example, the potential inhibitory effect on the growth of a malignant tumor grown as xenograft in a mouse model is described in Example 15 herein. Other examples of relevant cancer types, cell lines and rodent models that may be employed include:

#### *Hematopoietic cells*

Burkitt's lymphoma: Daudi or Raji cell line xenografted in mice.

Leukemia: K562-ADM or MOLT-4 cell line xenografted in mice or rats.

Myeloma: RPMI 8226 cell line xenografted in mice.

#### *Lung carcinoma*

Carcinoma: NCI H460 cell line xenografted in mice or rats.

*Breast carcinoma*

Adenocarcinoma: MCF7 cell line xenografted in mice or rats.

*Pancreatic carcinoma*

Carcinoma: PANC-1 cell line xenografted in mice.

*Ovarian carcinoma*

Adenocarcinoma: OVCAR-3 cell line xenografted in mice or rats.

*Colon carcinoma*

Carcinoma: HT29 (HT29/P27) cell line xenografted in mice or rats.

*Melanoma*

Malignant melanoma: A375-SM cell line xenografted in mice or rats.

*Bladder cancer*

Transitional papilloma: RT4 cell line xenografted in mice.

*Stomach carcinoma*

Carcinoma: AGS cell line xenografted in mice.

The invention is further described in the following examples. The examples should not, in any manner, be understood as limiting the generality of the present specification and claims.

**MATERIALS AND METHODS****Materials**

HeLa cells – (available from American Type Culture Collection (ATCC))

ISRE-Luc (Stratagene, La Jolla USA)

pCDNA 3.1/hygro (Invitrogen, Carlsbad USA)

pGL3 basic vector (Promega)

Human genomic DNA (CloneTech, USA)

DMEM medium: Dulbecco's Modified Eagle Media (DMEM), 10% fetal bovine serum (available from Life Technologies A/S, Copenhagen, Denmark)

## Assays

### Interferon Assay Outline

It has previously been published that IFNB interacts with and activates Interferon type I receptors on HeLa cells. Consequently, transcription is activated at promoters containing an Interferon Stimulated Response Element (ISRE). It is thus possible to screen for agonists of interferon receptors by use of an ISRE coupled luciferase reporter gene (ISRE-luc) placed in HeLa cells.

### *Primary Assay*

HeLa cells are co-transfected with ISRE-Luc and pCDNA 3.1/hygro and foci (cell clones) are created by selection in DMEM media containing Hygromycin B. Cell clones are screened for luciferase activity in the presence or absence of IFNB. Those clones showing the highest ratio of stimulated to unstimulated luciferase activity are used in further assays.

To screen muteins, 15,000 cells/well are seeded in 96 well culture plates and incubated overnight in DMEM media. The next day muteins as well as a known standard are added to the cells in various concentrations. The plates are incubated for 6 hours at 37°C in a 5% CO<sub>2</sub> air atmosphere. LucLite substrate (Packard Bioscience, Groningen The Netherlands) is subsequently added to each well. Plates are sealed and luminescence measured on a TopCount luminometer (Packard) in SPC (single photon counting) mode. Each individual plate contains wells incubated with IFNB as a stimulated control and other wells containing normal media as an unstimulated control. The ratio between stimulated and unstimulated luciferase activity serves as an internal standard for both mutein activity and experiment-to-experiment variation.

### *Secondary Assay*

Currently, there are 18 non-allelic interferon  $\alpha$  genes and one IFNB gene. These proteins exhibit overlapping activities and thus it is critical to ensure that muteins retain the selectivity and specificity of IFNB.

The  $\beta$ -R1 gene is activated by IFNB but not by other interferons. The transcription of  $\beta$ -R1 thus serves as a second marker of IFNB activation and is used to ensure that muteins retain IFNB activity. A 300 bp promoter fragment of  $\beta$ -R1 shown to drive interferon sensitive

transcription (Rani. M.R. et al (1996) *JBC* 271 22878-22884) was isolated by PCR from human genomic DNA and inserted into the pGL3 basic vector (Promega). The resulting  $\beta$ -R1:luciferase gene is used in assays similar to the primary assay described above. In astrocytoma cells, the resulting  $\beta$ -R1:luciferase gene has been described to show 250 fold higher sensitivity to IFNB than to interferon  $\alpha$  (Rani et al. *op cit*).

#### *ELISA assay*

The concentration of IFNB is quantitated by use of a sandwich ELISA using two monoclonal antibodies specific for IFN- $\beta$ . Tests samples and recombinant human IFN- $\beta$  standard are added in 0.1 ml in concentrations from 10-0.25 ng/ml to microtiter plates, precoated with catching antibody. The plates are incubated at RT for 1 hr. Samples and standard are diluted in kit dilution buffer.

The plates are washed in the kit buffer and incubated with the biotinylated detecting antibody in 0.1 ml for 1 hr at room temperature. After another wash the streptavidin-horseradishperoxidase conjugate is added in 0.1 ml and incubated for 1 hr at room temperature.

The reaction is visualised by addition of 0.1 ml tetramethylbenzidine (TMB) substrate chromogen. The plates are incubated for 15 minutes in the dark at room temperature and the reaction is stopped by addition of stop solution. The absorbance is read at 450nm using an ELISA reader.

In an alternative assay format purified polyclonal rabbit antibodies specific for IFN- $\beta$  are used. These antibodies were used in a non-conjugated form as catching antibody, and in a biotinylated form as detecting antibody. Otherwise the assay was conducted as described for the monoclonal ELISA.

In a further assay format, the concentration of IFNB is quantitated by use of a commercial sandwich immunoassay (PBL Biomedical Laboratories, New Brunswick, NJ, USA). The kit is based on an ELISA with monoclonal mouse anti-IFN- $\beta$  antibodies for catching and detection of IFN- $\beta$  in test samples. The detecting antibody is conjugated to biotin. Otherwise the assay was conducted as described above.

#### *Receptor binding assay*

The receptor binding capability of a polypeptide or conjugate of the invention can be determined using the assay described in WO 95/25170 entitled "Analysis Of IFN- $\beta$ (Phe<sub>101</sub>) For Receptor Binding" (which is based on Daudi or A549 cells). Soluble domains of IFNAR1 and

IFNAR2 can be obtained essentially as described by Arduini et al., *Protein Science*, 1999, vol. 8, 1867-1877 or as described in Example 9 herein.

Alternatively, the receptor binding capability is determined using a crosslinking agent such as disuccinimidyl suberate (DSS) available from Pierce, Rockford, IL, USA as follows:

The polypeptide or conjugate is incubated with soluble IFNAR-2 receptor in the presence or absence of DSS in accordance with the manufacturer's instructions. Samples are separated by SDS-PAGE, and a western blot using anti-IFNB or anti-IFNAR2 antibodies is performed. The presence of a functional IFNB polypeptide/conjugate: receptor interaction is apparent by an increase in the molecular size of receptor and IFNB in the presence of DSS.

Furthermore, a crosslinking assay using a polypeptide or conjugate of the invention and both receptor subunits (IFNAR-1 and IFNAR-2) can establish Interferon receptor 1 binding ability. In this connection it has been published that IFNAR-1 binds only after an interferon  $\beta$ : IFNAR-2 complex is formed (Mogensen et al., *Journal of Interferon and Cytokine Research*, 19:1069-1098, 1999).

#### *In vitro immunogenicity tests of interferon $\beta$ polypeptides*

Reduced immunogenicity of an IFNB polypeptide is determined by use of an ELISA method measuring the immunoreactivity of the conjugate or polypeptide relative to a reference molecule or preparation. The reference molecule or preparation is normally a recombinant human IFNB preparation such as Avonex®, Rebif® or Betaseron®, or another recombinant human IFNB preparation produced by a method equivalent to the way these products are made. The ELISA method is based on antibodies from patients treated with one of these recombinant IFNB preparations. The immunogenicity is considered to be reduced when the IFNB polypeptide disclosed herein has a statistically significant lower response in the assay than the reference molecule or preparation.

Another method of determining immunogenicity is by use of sera from patients treated with IFNB (i.e. any commercial IFNB product) in an analogous manner to that described by Ross et al., *J. Clin Invest.* 95, 1974-78, 1995. In the antiviral neutralisation bioassay reduced immunogenicity results in reduced inhibition of a conjugate of the invention by patient sera compared to a wt IFNB reference molecule. Furthermore, in the biochemical IFN binding assay a less immunogenic conjugate is expected to bind to patient IgG to a lesser extent than reference IFNB molecules.



For the neutralisation assay, the reference and conjugate molecules are added in a concentration that produces approximately 80% virus protection in the antiviral neutralisation bioassay. The IFNB proteins are mixed with patient sera in various dilutions (starting at 1:20).

#### *Antiviral activity*

The antiviral bioassay is performed using A549 cells (CCL 185, American tissue culture collection) and Encephalomyocarditis (EMC) virus (VR-129B, American tissue culture collection).

The cells are seeded in 96 well tissue culture plates at a concentration of 10,000 cells/well and incubated at 37°C in a 5% CO<sub>2</sub> air atmosphere. A polypeptide or conjugate of the invention is added in concentrations from 100-0.0001 IU/mL in a total of 100 µl DMEM medium containing fetal calf serum and antibiotics.

After 24 hours the medium is removed and 0.1 mL fresh medium containing EMC virus is added to each well. The EMC virus is added in a concentration that causes 100% cell death in IFN-β free cell cultures after 24 hours.

After another 24 hrs, the antiviral effect of the polypeptide or conjugate is measured using the WST-1 assay. 0.01 mL WST-1 (WST-1 cell proliferation agent, Roche Diagnostics GmbH, Mannheim, Germany) is added to 0.1 mL culture and incubated for ½-2 hours at 37°C in a 5% CO<sub>2</sub> air atmosphere. The cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells results in the formation of formazan that is quantified by measuring the absorbance at 450 nm.

#### Neutralisation assays

To evaluate the neutralisation by antibodies, animal or patient serum samples two assay formats are used.

#### *Neutralisation of activity antiviral activity assay*

The neutralising effect of IFNB sera is evaluated using the antiviral activity assay described above.

Serum samples were diluted and mixed with a fixed amount (typically 10-20 IU/ml) of rhIFNB or a conjugate as described herein for 1 hr at 37°C. The mixture was then added to the antiviral assay in a total of 100 µl DMEM medium containing serum and antibiotics.

After 24 hours the medium was removed and 0.1 ml fresh medium containing EMC virus was added to each well. The EMC virus was added in a concentration that causes 100% cell death in IFNB-free cell cultures after 24 hours.

After another 24 hrs, the antiviral effect of the polypeptide was measured using the WST-1 assay. 0.01 ml WST-1 (WST-1 cell proliferation agent, Roche Diagnostics GmbH, Mannheim, Germany) was added to 0.1 ml culture and incubated for ½-2 hours at 37°C in a 5% CO<sub>2</sub> air atmosphere. The cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases in viable cells results in the formation of formazan which was quantified by measuring the absorbance at 450 nm. The neutralising of the serum sample was calculated as the dilution of serum inducing a 50% reduction in virus protection by the added IFNB.

*Neutralisation of activity in Interferon Stimulated Response Element (ISRE) assay*

The IFNB neutralising effect of anti-IFNB sera are analysed using the ISRE-Luciferase activity assay.

Sera from IFNB treated patients or from immunised animals are used. Sera are added either in a fixed concentration (dilution 1:20-1:500 (pt sera) or 20-600 ng/mL (animal sera)) or in five-fold serial dilutions of sera starting at 1/20 (pt sera) or 600 ng/mL (animal sera). IFNB is added either in five fold-dilutions starting at 25.000 IU/mL or in a fixed concentration (0.1-10 IU/mL) in a total volume of 80 µl DMEM medium + 10% FCS. The sera are incubated for 1 hr at 37°C with IFN-β.

The samples are then transferred to 96 well tissue culture plates containing HeLa cells transfected with ISRE-Luc grown from 24 hrs before (15,000 cells/well) in DMEM media. The cultures are incubated for 6 hours at 37°C in a 5% CO<sub>2</sub> air atmosphere. LucLite substrate (Packard Bioscience, Groningen, The Netherlands) is subsequently added to each well. Plates are sealed and luminescence measured on a TopCount luminometer (Packard) in SPC (single photon counting) mode.

When IFNB samples are titrated in the presence of a fixed amount of serum, the neutralising effect was defined as fold inhibition (FI) quantified as EC<sub>50</sub>(w. serum)/EC<sub>50</sub> (w/o serum). The reduction of antibody neutralisation of IFNB variant proteins is defined as

$$\left(1 - \frac{\text{FI variant}}{\text{FI wt}}\right) \times 100\%$$

### Biological half-life measurements

Measurement of biological half-life can be carried out in a number of ways described in the literature. One method is described by Munafo et al., *European Journal of Neurology* 1998, vol 5 No2 p 187-193), who used an ELISA method to detect serum levels of IFNB after subcutaneous and intramuscular administration of IFNB.

The rapid decrease of IFNB serum concentrations after i.v. administration has made it important to evaluate biological responses to IFNB treatment. However it is contemplated that the conjugates of the present invention will have prolonged serum half lives also after i.v. administration making it possible to measure by e.g. an ELISA method or by the primary screening assay.

Different pharmacodynamic markers (e.g. serum neopterin and beta2 microglobulin) have also been studied (*Clin Drug Invest* (1999) 18(1):27-34). These can equally well be used to evaluate prolonged biological effect. These experiments may also be carried out in suitable animal species, e.g. rats.

Assays to assess the biological effects of IFNB such as antiviral, antiproliferative and immunomodulatory effects (as described in e.g. *Annals of Neurology* 1995 vol 37 No 1 p 7-15) can be used together with the primary and secondary screening assays described herein to evaluate the biological efficacy of the conjugate in comparison to wild type IFNB.

Finally an animal model such as the commonly used experimental autoimmune encephalomyelitis (EAE) model can be used to establish efficacy of a conjugate or polypeptide of the invention. In the EAE model immunization with myelin or myelin-derived proteins elicits a disease mimicking the majority of the inflammatory and neurologic features of multiple sclerosis in humans. EAE has been used in mice, rats, rabbits, and marmosets (Cannella et al. *PNAS*, 95, 10100-5, 1998, Zaprianova et al. *Morfologija*, 112, 25-8, 1997, Hassouna et al. *J. Urology*, 130, 806-10, 1983, Genain & Hauser *J. Mol. Med.* 75, 187-97, 1997). Other models include Theiler's murine encephalomyelitis virus (TMEV) model (Murray et al. *J. Neurosci.* 18, 7306-14, 1998).

### Accessible Surface Area (ASA)

The computer program Access (B. Lee and F.M. Richards, *J. Mol. Biol.* 55: 379-400 (1971)) version 2 (Copyright (c) 1983 Yale University) are used to compute the accessible surface area (ASA) of the individual atoms in the structure. This method typically uses a probe-

size of 1.4Å and defines the Accessible Surface Area (ASA) as the area formed by the centre of the probe. Prior to this calculation all water molecules and all hydrogen atoms are removed from the coordinate set, as are other atoms not directly related to the protein. Alternative programs are available for computing ASA, e.g. the program WhatIf G.Vriend, *J. Mol. Graph.* (1990) 8, 52-56, electronically available at the WWW interface on <http://swift.embl-heidelberg.de/servers2/> (R.Rodriguez et.al. *CABIOS* (1998) 14, 523-528.) using the option *Accessibility* to calculate the accessible molecular surface.

#### Fractional ASA of side chain

The fractional ASA of the side chain atoms is computed by division of the sum of the ASA of the atoms in the side chain with a value representing the ASA of the side chain atoms of that residue type in an extended ALA-x-ALA tripeptide. See Hubbard, Campbell & Thornton (1991) *J.Mol.Biol.* 220,507-530. For this example the CA atom is regarded as a part of the side chain of Glycine residues but not for the remaining residues. The following table indicates the 100% ASA standard for the side chain:

Ala	69.23 Å <sup>2</sup>
Arg	200.35 Å <sup>2</sup>
Asn	106.25 Å <sup>2</sup>
Asp	102.06 Å <sup>2</sup>
Cys	96.69 Å <sup>2</sup>
Gln	140.58 Å <sup>2</sup>
Glu	134.61 Å <sup>2</sup>
Gly	32.28 Å <sup>2</sup>
His	147.00 Å <sup>2</sup>
Ile	137.91 Å <sup>2</sup>
Leu	140.76 Å <sup>2</sup>
Lys	162.50 Å <sup>2</sup>
Met	156.08 Å <sup>2</sup>
Phe	163.90 Å <sup>2</sup>
Pro	119.65 Å <sup>2</sup>
Ser	78.16 Å <sup>2</sup>
Thr	101.67 Å <sup>2</sup>

44

Trp	210.89 Å <sup>2</sup>
Tyr	176.61 Å <sup>2</sup>
Val	114.14 Å <sup>2</sup>

#### Determining surface exposed amino acid residues

The three-dimensional crystal structure of human IFNB at 2.2 Å resolution (Karpusas et al. *Proc. Nat. Acad. Sci. USA* (1997) 94:11813-11818 is available from the Protein Data Bank (PDB) (Bernstein et.al. *J. Mol. Biol.* (1977) 112 pp. 535) and electronically available via The Research Collaboratory for Structural Bioinformatics PDB at <http://www.pdb.org/> under accession code 1AU1. This crystal structure contain two independent molecules of human IFNB in this example the A molecule is used.

#### Surface exposure:

Using the WhatIf program as described above the following residues were found to have zero surface accessibility for their side chain atoms (for Gly the accessibility of the CA atom is used): G7, N14, C17, L21, I44, A55, A56, T58, I59, M62, L63, L98, L122, Y125, I129, L133, A142, W143, V146, I150, N153, I157, L160, T161, and L164.

#### Fractional surface exposure

For further analysis it was necessary to remodel the side chains of residues R71, R113, K115, L116, M117 due to steric clashes. The remodelling was done using Modeler 98, MSI INC. Performing fractional ASA calculations using the Access computer program on the remodelled IFNB molecule (only including the amino acid residues and excluding the N-linked sugar moiety) resulted in the following residues having more than 25% of their side chain exposed to the surface: S2, N4, L5, F8, L9, R11, S12, F15, Q16 Q18, K19, W22, Q23, G26, R27, L28, E29, Y30, L32, K33, R35, M36, N37, D39, E42, K45, Q46, L47, Q48, Q49, Q51, K52, Q64, A68, R71, Q72, D73, S75, S76, G78, N80, E81, T82, E85, N86, A89, Y92, H93, N96, H97, K99, T100, E103, E104, K105, E107, K108, E109, D110, F111, R113, G114, K115, L116, S119, L120, H121, K123, R124, G127, R128, L130, H131, K134, A135, K136, E137, Y138, S139, H140, V148, R152, Y155, N158, G162, Y163, R165, and N166. The following residues have more than 50% of their side chain exposed to the surface: N4, L5, F8, S12, F15, Q16, K19, W22, G26, R27, E29, Y30, K33, R35, N37, D39, E42, Q46, Q48, Q49, Q51, K52, R71, D73, S75, G78, N80, E81, T82, E85, N86, A89, Y92, H93, K99, T100, E103, E104,

E107, K108, D110, F111, L116, K123, R124, G127, H131, K134, E137, V148, Y155, R165, and N166.

## EXAMPLES

### Example 1

#### *Design of an expression cassette for expression of IFNB in mammalian and insect cells*

The DNA sequence, GenBank accession number M28622 (shown in SEQ ID NO 1), encompassing a full length cDNA encoding human IFNB with its native signal peptide, was modified in order to facilitate high expression in mammalian cells. First the ATG start codon context was modified according to the Kozak consensus sequence (Kozak, M. *J Mol Biol* 1987 Aug 20; 196(4):947-50), such that there is a perfect match to the consensus sequence upstream of the ATG start codon. Secondly the codons of the native human IFNB was modified by making a bias in the codon usage towards the codons frequently used in highly expressed human genes. Subsequently, certain nucleotides in the sequence were substituted with others in order to introduce recognition sites for DNA restriction endonucleases (this allows for easier modification of the DNA sequence later). Primers were designed such that the gene could be synthesised:

#### *CBProFpr1:*

5'GGCTAGCGTTTAAACTTAAGCTTCGCCACCATGACCAACAAGTGCCTGCTCCAGATCGCCCTGCTCCTGT-3',

#### *CBProFpr2:*

5'ACAACCTGCTCGGCTTCCTGCAGAGGAGTTCGAACTCCAGTGCCAGAAGCTCCTGTGGCAGCTGAACGG-3',

#### *CBProFpr3:*

5'GAACTTCGACATCCCCGAGGAAATCAAGCAGCTGCAGCAGTTCAGAAGGAGGACGCCGCTCTGACCATC-3',

#### *CBProFpr4*

5'TTCCGCCAGGACTCCAGCTCCACCGGTTGGAACGAGACCATCGTGGAGAACCTGCTGGCCAACGTGTACC-3',

#### *CBProFpr5*

5'AGGAGAAGCTGGAGAAGGAGGACTTCACCCGCGGCAAGCTGATGAGCTCCCTGCACCTGAAGCGCTACTA-3',

*CBProFpr6*

5'GGAGTACAGCCACTGCGCCTGGACCATCGTACGCGTGGAGATCCTGCGCAACTTC  
TACTTCATCAACCGC-3',

*CBProFpr9*

5'CACCACACTGGACTAGTGGATCCTTATCAGTTGCGCAGGTAGCCGGTCAGGCGGT  
TGATGAAGTAGAAGT-3',

*CBProFpr10*

5'AGGCGCAGTGGCTGTACTCCTTGGCCTTCAGGTAGTGCAGGATGCGGCCATAGTA  
GCGCTTCAGGTGCAG-3',

*CBProFpr11*

5'CTCCTTCTCCAGCTTCTCCTCCAGCACGGTCTTCAGGTGGTTGATCTGGTGGTACA  
CGTTGGCCAGCAGG-3',

*CBProFpr12*

5'GAGCTGGAGTCCTGGCGGAAGATGGCGAAGATGTTCTGCAGCATCTCGTAGATG  
GTCAGAGCGGCGTCCT-3',

*CBProFpr13*

5'CCTCGGGGATGTCGAAGTTCATCCTGTCCTTCAGGCAGTACTCCAGGCGCCCGTT  
CAGCTGCCACAGGAG-3',

*CBProFpr14*

5'CAGGAAGCCGAGCAGGTTGTAGCTCATCGATAGGGCCGTGGTGCTGAAGCACAG  
GAGCAGGGCGATCTGG-3',

The primers were assembled to the synthetic gene by one step PCR using Platinum *Pfx*-polymerase kit (Life Technologies) and standard three step PCR cycling parameters. The assembled gene was amplified by PCR using the same conditions.

A cDNA encoding a N-terminal extended form of human IFNB was synthesised using the same PCR conditions as described above but with the primers CBProFpr1 and -14 substituted with the primers:

*CBProFpr7*

5'CTGCTCCAGATCGCCCTGCTCCTGTGCTTCAGCACACGGCCCTATCGATGAAGC  
ACCAGCACCATC-3',

*CBProFpr8*

5'CACTGCTTACTGGCTTATCGAAATTAATACGACTCACTATAGGGAGACCCAAGCT  
GGCTAGCGTTTAAAC-3',

*CBProFpr15*

5'CAGGAAGCCGAGCAGGTTGTAGCTCATCTGTTGGTGTGATGTTGGTGCTGATGC  
TGGTGCTGGTGCTTC-3',

*CBProFpr16*

5'AGCAGGGCGATCTGGAGCAGGCACTTGTGGTCATGGTGGCGAAGCTTAAGTTTA  
AACGCTAGCCAGCTT-3',

in order to incorporate a purification TAG in the IFNB molecule.

The synthesised genes were cloned into pcDNA3.1/Hygro (Invitrogen) between the *HindIII* site at the 5' end and the *BamHI* at the 3', resulting in pCBProF1 and pCBProF2.

The synthetic intron from pCI-Neo (Promega) was amplified using standard PCR conditions as described above and the primers:

*CBProFpr37* 5'-CCGTCAGATCCTAGGCTAGCTTATTGCGGTAGTTTATCAC-3',

*CBProFpr38* 5'-GAGCTCGGTACCAAGCTTTTAAGAGCTGTAAT-3',

resulting in a 332 bp PCR fragment which was cut with *NheI* and *HindIII* and inserted in the 5'UTR of the plasmids pCBProF1 and pCBProF2 resulting in pCBProF4 and pCBProF5.

Codons for individual amino acids were changed by amplifying relevant regions of the coding region by PCR in such a way that the PCR introduced changes in the sequence can be introduced in the expression plasmids by classical cloning techniques. E.g. the primers:

*Lys45arg-5'primer* (*NarI/KasI*):

5'GCTGAACGGGCGCCTGGAGTACTGCCTGAAGGACAGGATGAACTTCGACATCCC  
CGAGGAAATCCGCCAGCTGCAGC-3',

*Lys45mut-3'primer* (*BsiWI*): 5'TCTCCACGCGTACGATGGTCCAGGCGCAGTGGCTG-3',

were used to introduce a K45R substitution in the PCR-fragment spanning the region from position 1055 to 1243 in pCBProF1. Both the PCR fragment and pCBProF1 was cut with *NarI* and *BsiWI* which are both unique. The PCR fragment and the vector backbone of pCBProF1 are purified and ligated resulting in substitution of the Lys45 codon AAG with the Arg codon CGC in pCBProF1.

Furthermore, SOE (sequence overhang extension) PCR was used for introduction of amino acid substitutions. In the SOE-PCR both the N-terminal part and the C-terminal part of the IFNB molecule were first amplified in individual primary PCRs.

For these primary PCRs the central complementary primers were synthesised such that the codon(s) for the amino acid(s) to be substituted is/are changed to the desired codon(s). The terminal primers were standard primers defining the N- and C-terminal of the IFNB molecule



respectively. Further the terminal primers provided a restriction enzyme site enabling subsequent cloning of the full-length PCR product. Thus, the central (nonsense) primer and the N-terminal (sense) primer were used to amplify the N-terminal part of the IFNB coding region in one of the primary PCRs and equivalently for the C-terminal part. Once amplified the N- and C-terminal parts are assembled into the full-length product in a secondary PCR and cloned into a modified version of pCDNA3.1/Hygro as described above. For instance, the following primers were used to introduce the mutations for the substitutions F111N and R113T:

*CBProFprimer9*(Sense):

CACCACACTGGACTAGTGGATCCTTATCAGTTGCGCAGGTAGCCGGTCAGGCGGTT  
G ATG AAGTAGAAGT,

*CBProFprimer231*(Antisense):

CATCAGCTTGCCGGTGGTGTGTCCTCCTTC,

*CBProFprimer230* (Sense):

GAAGGAGGACAACACCACCGGCAAGCTGATG,

*CBProFprimer42* (Antisense):

CACACTGGACTAGTAAGCTTTTATCAGTTGCGCAGGTAGC,

Furthermore, in cases where the introduced mutation(s) were sufficiently close to a unique restriction endo-nuclease site in the expression plasmid variant genes were constructed using construction procedure encompassing a single PCR step and a subsequent cloning. For instance, the substitution K19R was introduced by use of the PCR primer:

*CBProFpr58*:

GAGGAGTTCGAACTTCCAGTGCCAGCGCCTCCTGTGGCAGCTGAACG, and

*CBProFprimer9*:

The PCR product was subsequently cloned using the restriction endonuclease sites *Bsi*WI and *Bst*BI.

## Example 2

### *Construction and expression of the [F111N+R113T]hIFN- $\beta$ variant*

In order to insert an extra N-linked glycosylation site at position 111 in wild-type human IFNB, the synthetic gene (*hinf*- $\beta$ ) encoding wild-type human IFNB (described in Example 1) was altered by site-directed PCR mutagenesis. Using BIO-X-ACT (Bioline, UK) and the plasmid PF050 [*hinf*- $\beta$ ]/pcDNA3.1(-)Hygro/Intron (a derivative of pcDNA3.1(-)Hygro (Invitrogen, USA) in which a chimeric intron obtained from pCI-neo (Promega, USA) had been

inserted between the BamHI and NheI sites in the MCS of the vector] as template, two PCR reactions were performed with two overlapping primer-sets [*CB41* (5'-TTTAAACTGGATCCAGCCACCATGACCAACAAG-3') / *CB55* (5'-CGGCCATAGT AGCGCTTCAGGTGCAGGGAGCTCATCAGCTTGCCGGTGGTGTGTCCTCCTTC-3') and *CB42* / *CB86* (5'-GAAGGAGGACAACACCACCGGCAAGCTGATGAGCTCCCTGCACCTGAAGCGCTAC TATGGCC G-3') resulting in two fragments of 446 and 184 base pairs, respectively. These two fragments were assembled in a third PCR with the flanking primers *CB41* and *CB42*. The resulting gene was inserted into the mammalian expression vector pcDNA3.1(-)Hygro/Intron and confirmed by DNA sequencing to have the correct base changes leading to the substitutions F111N and R113T in hIFN- $\beta$  (plasmid designated PF085).

To test the activity of the [F111N+ R113T]hIFN- $\beta$  variant, PF085 was transfected into the CHO K1 cell line (ATCC #CCL-61) by use of Lipofectamine 2000 (Life Technologies, USA) as transfection agent. 24 hours later the culture medium was harvested and assayed for IFN- $\beta$  activity/concentration:

Activity:	56046 IU/ml [primary assay]
ELISA:	80 ng/ml
Specific activity:	$7 \times 10^8$ IU/mg

As can be seen, the [F111N+R113T]hIFN- $\beta$  variant has a very high specific activity, about twice the specific activity of wt hIFN- $\beta$ .

### Example 3

#### *Construction and expression of the [Q49N+Q51T]hIFN- $\beta$ variant*

Analogously to what is described in Example 2 an extra N-linked glycosylation site was introduced in position 49 by means of the substitutions Q49N and Q51T. Using PF043 (*hinf*- $\beta$  / pcDNA3.1 (Invitrogen, USA)) as template, two PCR reactions were performed with two overlapping primer-sets [*PBR7* / *PBR78* (5'-GGCGTCCTCCTTGGTGAAGTTCTGCAGCTG-3') and *PBR8* (5'-ATATATCCCAAGCTTTTATCAGTTGCGCAGGTAGCCGGT-3') / *PBR77* (5'-CAGCTGCAGAACTTCACCAAGGAGGACGCC-3') resulting in two fragments of 228 and 369 base pairs, respectively. These two fragments were assembled in a third PCR with the flanking primers *PBR7* and *PBR8*. The resulting gene was inserted into the mammalian

expression vector pcDNA3.1(-)Hygro/Intron and confirmed by DNA sequencing to have the correct base changes leading to [Q49N,Q51T]hIFN- $\beta$  (plasmid designated PF104).

To test the activity of the [Q49N+Q51T]hIFN- $\beta$  variant, PF104 was transfected into the CHO K1 cell line by use of Lipofectamine 2000 (Life Technologies, USA) as transfection agent. 24 hours later the culture medium was harvested and assayed for IFN- $\beta$  activity/concentration:

Activity:	17639 IU/ml [primary assay]
ELISA:	10 ng/ml
Specific activity:	$1.7 \times 10^9$ IU/mg

As can be seen, the [Q49N+Q51T]hIFN- $\beta$  variant has a high specific activity

#### Example 4

##### *Construction and expression of the [Q49N+Q51T+F111N+R113T]hIFN- $\beta$ variant*

The additional glycosylation sites described in Examples 2 and 3 were introduced into human IFNB by means of the substitutions Q49N, Q51T, F111N, and R113T.

Using PF085 (described in example 5) as template, two PCR reactions were performed with two overlapping primer-sets [PBR89

(5'CGCGGATCCAGCCACCATGACCAACAAGTGCCTG)/

PBR78 and PBR8/PBR77] resulting in two fragments of 228 and 369 base pairs, respectively.

These two fragments were assembled in a third PCR with the flanking primers PBR89 and PBR8. The resulting gene was inserted into the mammalian expression vector pcDNA3.1(-)Hygro/Intron and confirmed by sequencing to have the correct base changes leading to [Q49N, Q51T, F111N, R113T] hIFN- $\beta$  (plasmid designated PF123).

PF123 was transfected into CHO K1 cells by use of Fugene 6 (Roche) as transfection agent. 24 hours later the culture medium was harvested and assayed for IFN- $\beta$  activity/concentration:

Activity:	29401 IU/ml [primary assay]
ELISA:	14 ng/ml
Specific activity:	$2.1 \times 10^9$ IU/ml

As can be seen, the [Q49N+Q51T+ F111N+ R113T]hIFN- $\beta$  variant also has a high specific activity.

The variant was found to have receptor binding activity in the receptor binding assay described in the Materials and Methods section, which is based on the use of the crosslinking agent DSS.

### Example 5

#### *Production of [Q49N+Q51T+F111N+R113T]hIFN- $\beta$ in Roller Bottles*

A CHOK1 sub-clone (5/G-10) producing the [Q49N+Q51T+F111N+R113T] glycosylation variant was seeded into 2 roller bottles, each with an expanded surface of 1700 cm<sup>2</sup> (Corning, USA), in 200 ml DMEM/F-12 medium (Life Technologies; Cat. # 31330) supplemented with 10% FBS and penicillin/streptomycin (P/S). After 2 days the medium was exchanged. After another 2 days the two roller bottles were nearly 100% confluent and the medium was shifted to 300 ml serum-free UltraCHO medium (BioWhittaker; Cat. # 12-724) supplemented with 1/500 EX-CYTE (Serologicals Proteins; Cat. # 81129N) and P/S. Growing the cells in this medium promotes a higher cell mass, higher than can be achieved in the serum containing medium. After 2 days the medium was renewed. After another 2 days the medium was shifted to the production medium: DMEM/F-12 medium (Life Technologies; Cat. # 21041) supplemented with 1/100 ITSA (Life Technologies; Cat. # 51300-044) [ITSA stands for Insulin (1.0 g/L) – Transferrin (0.55 g/L) – Selenium (0.67 mg/L) supplement for Adherent cultures], 1/500 EC-CYTE and P/S. The harvested media from the two roller bottles were pooled before a medium sample was taken out for IFNB activity determination.

### Example 6

#### *Production, purification, and PEGylation of the [K19R+ K45R+ K123R]hIFN- $\beta$ variant*

To end up with 100 ml serum-free medium containing the IFNB variant K19R+K45R+K123R, 3 T-175 flasks were seeded with COS-7 cells in DMEM medium (Life technologies; Cat. # 21969-035) supplemented with 10% FBS plus Glutamine and penicillin/streptomycin. On the day of transfection (at nearly 100% confluency) the medium was renewed with 30 ml fresh medium 4 – 5 hours before the transfection. To prepare the transfection, 1890  $\mu$ l DMEM medium without supplements was aliquoted into a 14 ml polypropylene tube (Corning). 210  $\mu$ l Fugene 6 (Roche) was added directly into the medium and incubated for 5 min at RT. In the meantime 168  $\mu$ g plasmid DNA ([K19R, K45R, K123R]IFN- $\beta$ /pcDNA3.1(-)Hygro; PF #161) was aliquoted into another 14 ml polypropylene tube. After 5 min incubation the Fugene 6 mix was added directly to the DNA solution and

incubated for 15 min at RT. After incubation about 700  $\mu$ l was added drop wise to each of the three cell media.

Next day the transfection medium was substituted with 35 ml serum-free production medium. The serum-free medium is based on DMEM medium (Life Technologies; Cat. # 31053-028) supplemented with Glutamine, Sodium Pyruvate, penicillin/streptomycin, 1% ITSA (Life Technologies; Cat. # 51300-044), and 0.2% Ex-Cyte (Serologicals Proteins; Cat. # 81-129). Before the production medium was added the cell layers were washed two times in the DMEM medium without additives.

Three days post-transfection the 100 ml serum-free medium was harvested for purification and PEGylation of the IFNB variant.

pH was adjusted to 6.8 and conductivity adjusted to < 10 mS/cm with Milli Q water. Then the broth was batch adsorbed to 1 ml SP 550 cation exchange resin (TosoHaas) preequilibrated with buffer A (20 mM phosphate, 100 mM NaCl, pH 7). After 2 h rotation end over end, the resin was allowed to sediment and transferred to a column. The resin was washed with 5 column volumes buffer A and eluted with 2 ml buffer B (20 mM phosphate, 800 mM NaCl, pH 7). The eluate was concentrated to 500  $\mu$ l on VivaSpin (cutoff 10 kDa) after addition of 5 % ethyleneglycol. The concentrate was adjusted to 50 mM phosphate, 0.3 M NaCl, 20 % ethyleneglycol, pH 8 in a final volume of 2 ml and further concentrated to 0.5 ml.

The final concentrate was PEGylated as follows: to 100  $\mu$ l of the final concentrate, 25  $\mu$ l of activated mPEG-SPA (5000 kDa, Shearwater, Alabama) freshly prepared in phosphate buffer, pH 8 were added to make final concentrations of activated PEG of 0, 5, 10, 25 or 50 mg/ml. The reaction was allowed to proceed for 30 min at room temperature and then quenched by addition of 50 mM glycine buffer. Samples were frozen immediately at  $-80^{\circ}\text{C}$  and bioactivity was measured as described (Primary Assay). Western blots of each sample were performed in order to evaluate the amount of unreacted IFNB variant present in the PEGylated sample.

Results demonstrate that at 25 mg activated PEG/ml, nonPEGylated IFNB variant was absent as judged by western blot and the variant retained 50 % of its bioactivity compared to the control sample (treated identically, but with 0 mg/ml activated PEG).

**Example 7***Variants having increased glycosylation in position 49*

The inserted N-linked glycosylation site in position 49 of the IFNB variant [Q49N+Q51T] described in Example 3 is used only about 60%. In order to increase the amount of attached carbohydrate the glutamine residue at position 48 was substituted with phenylalanine (Q48F), valine (Q48V), and tryptophan (Q48W) by site-directed PCR mutagenesis. Using BIO-X-ACT (Bioline, UK) and PF185 (PF185 contains the same cDNA sequence as PF104, described in Example 4, despite the fact that a Kozak sequence has been inserted in front of the start ATG) as template, PCR reactions were performed with overlapping primer-sets:

Q48F+Q49N+Q51T

PBR89 (5'CGCGGATCCAGCCACCATGACCAACAAGTGCCTG)/PBR148 (5'GTCCTCCTTGGTGAAGTTGAACAGCTGCTT) and PBR8 ((5'-ATATATCCCAAGCTTTTATCAGTTGCGCAGGTAGCCGGT-3'))/PBR147 (5'AAGCAGCTGTTCAACTTCACCAAGGAGGAC)

Q48V+Q49N+Q51T

PBR89 (5'CGCGGATCCAGCCACCATGACCAACAAGTGCCTG)/PBR150 (5'GTCCTCCTTGGTGAAGTTTACCAGCTGCTT) and PBR8 /PBR149 (5'AAGCAGCTGGTGAAGTTCACCAAGGAGGAC)

Q48W+Q49N+Q51T

PBR89 (5'CGCGGATCCAGCCACCATGACCAACAAGTGCCTG)/PBR152 (5'GTCCTCCTTGGTGAAGTTCCACAGCTGCTT) and PBR8 /PBR151 (5'AAGCAGCTGTGGAAGTTCACCAAGGAGGAC)

The fragments were assembled in PCR reactions with the flanking primers PBR89 and PBR8. The resulting genes were inserted into the mammalian expression vector pcDNA3.1 (-Hygro/Intron and confirmed by sequencing to have the correct base changes leading to [Q48F+Q49N+Q51T]hIFN- $\beta$  (plasmid designated PF305), [Q48V+Q49N+Q51T]hIFN- $\beta$  (plasmid designated PF306) and [Q48W+Q49N+Q51T]hIFN- $\beta$  (plasmid designated PF307), respectively. PF305, PF306, PF307, and PF185 were transfected into CHO K1 cells by use of

Fugene 6 (Roche) as transfection agent. 24 hours later the culture medium was harvested and assayed for IFN- $\beta$  activity:

PF185	134713 IU/ml
PF305	53122 IU/ml
PF306	65949 IU/ml
PF307	45076 IU/ml

In order to evaluate the amount of attached carbohydrate in the three new glycosylation variants a Western blot was performed with equal amount of activity in each lane. From the Western blot it could be concluded that the amino acid substitutions (Q48F, Q48V, Q48W) all lead to increased glycosylation in position 49.

In another experiment it was seen that insertion of tyrosine in position 48 lead to increased glycosylation in position 49.

### Example 8

#### *Variants having increased glycosylation in position 111*

The inserted N-linked glycosylation site at position 111 in the IFNB variant [F111N+R113T] described in Example 2 is used only about 50%. In order to increase the amount of attached carbohydrate the aspartic acid residue at position 110 was substituted with phenylalanine (D110F) and valine (D110V) by site-directed PCR mutagenesis. Using BIO-X-ACT (Bioline, UK) and PF085 (described in Example 2) as template, PCR reactions were performed with overlapping primer-sets:

#### D110F+F111N+R113T

PBR89 (5'CGCGGATCCAGCCACCATGACCAACAAGTGCCTG)/PBR154  
(5'CAGCTTGCCGGTGGTGTGTAACCTCTCTC) and PBR8 /PBR153  
(GAGAAGGAGTTCAACACCACCGCAAGCTG)

#### D110V+F111N+R113T

PBR89 (5'CGCGGATCCAGCCACCATGACCAACAAGTGCCTG)/PBR156  
(5'CAGCTTGCCGGTGGTGTTCACCTCTCTC) and PBR 8 /PBR 155  
(5'GAGAAGGAGGTGAACACCACCGCAAGCTG)

The fragments were assembled in PCR reactions with the flanking primers PBR89 and PBR8. The resulting genes were inserted into the mammalian expression vector pcDNA3.1 (-)Hygro/Intron and confirmed by sequencing to have the correct base changes leading to

[D110F+F111N+R113T]hIFN- $\beta$  (plasmid designated PF308) and [D110V+F111N+R113T]hIFN- $\beta$  (plasmid designated PF309), respectively.

PF308, PF309 and PF085 (encoding [F111N+R113T]hIFN- $\beta$ ) were transfected into CHO K1 cells by use of Fugene 6 (Roche) as transfection agent. 24 hours later the culture medium was harvested and assayed for IFN- $\beta$  activity:

PF085	58615 IU/ml
PF308	50900 IU/ml
PF309	15063 IU/ml

In order to evaluate the amount of attached carbohydrate in the two new glycosylation variants a Western blot was performed with equal amount of activity in each lane. From the Western blot it could be concluded that the amino acid substitutions (D110F and D110V) in front of the introduced glycosylation site (F111N, R113T) lead to a significantly increased glycosylation in position 110.

In another experiment it was seen that insertion of tyrosine in position 110 lead to increased glycosylation in position 111.

### Example 9

#### *Separation of glycoforms*

Hydroxyapatite chromatography is an efficient means for separation of IFNB glycoforms and e.g. obtain glycoforms with fully utilized glycosylation sites. This is illustrated in the present example.

The IFNB variant [Q49N+Q51T+F111N+R113T] produced as described in Example 4 was purified in a three-step procedure:

The harvested media from roller bottles was centrifuged and filtered through a 0.22  $\mu$ m filter (PVDF). The filtrated media was diafiltrated on a Vivaflow 200 system equipped with a polyethersulfon membrane with cut off 10000 and applied to a S-Sepharose column (Pharmacia) equilibrated with 50 mM sodium acetate, 50 mM sodium chloride, pH 5.5. The interferon variant bound to the column was eluted with 50 mM sodium acetate, 0.5 M sodium chloride, pH 5.5. The concentration of sodium chloride in the eluate from the S-Sepharose column was adjusted to 1.0 M and the sample was applied on a Phenyl-Sepharose High Performance column (Pharmacia) equilibrated with 50 mM sodium acetate, 1.0 M sodium chloride, pH 5.5. Following application the column was washed with Milli Q water. The IFNB



variant was eluted with a gradient from Milli Q water to 60% ethylene glycol, 50 mM sodium acetate, pH 5.5 in 30 column volumes. Fractions containing fully glycosylated IFNB variant were collected and the buffer in the eluate was changed to 15 mM sodium phosphate buffer, pH 7.2. The sample was applied on a hydroxyapatite column (CHT II, Ceramic hydroxyapatite, Type II, Biorad) equilibrated with 15 mM sodium phosphate. The fully glycosylated form passed through the column whereas the underglycosylated form with one extra site used bound to the column and was eluted with a linear sodium phosphate gradient from 15 mM to 200 mM in 20 column volumes.

The purity of fully glycosylated [Q49N+Q51T+F111N+R113T] variant was judged to be higher than 95% based on SDS-PAGE.

### Example 10

#### *PEGylation IFNB glycosylation variants*

A fresh stock solution of SCM-PEG (succinimidyl ester of carboxymethylated PEG from Shearwater, Alabama, 5 kD or 12 kD) was prepared in methanol before each experiment.

100 microliter of a 0.3 mg/ml solution of the glycovariant [Q49N+Q51T+F111N+R113T]hIFN- $\beta$  in 50 mM sodium phosphate, 100 mM sodium chloride, pH 7.0 were PEGylated with SCM-PEG, 5 kD or 12 kD, with two times molar surplus of PEG to possible PEGylation sites, i.e. lysines and N-terminus. After incubation for 30 min at room temperature, the reaction was quenched by addition of 5  $\mu$ l 20 mM glycine, pH 8.0. At this stage, the reaction mixture contained a minor part of unmodified protein judged by SDS-PAGE.

*In vitro* testing using the primary screening assay demonstrated that the pegylated material retained 40% activity with 1-3 groups of 12 kD PEG attached. With 1-3 groups of 5 kD PEG attached the retained bioactivity was 25%.

In another experiment 50  $\mu$ l of purified [Q49N+Q51T+F111N+R113T+K19R+K45R+K123R]hIFN- $\beta$  with a protein concentration of 0.1 mg/ml was PEGylated in 50 mM sodium phosphate, 100 mM sodium chloride, pH 8.0 with SCM-PEG, 5 kD, with 20 times molar excess of PEG to possible PEGylation sites, i.e. lysines and N-terminus. After incubation for 30 min at room temperature, the reaction was quenched by addition of 5  $\mu$ l 20 mM glycine, pH 8.0. At this stage, the reaction mixture contained a minor part of unmodified protein judged by SDS-PAGE.

*In vitro* testing using the primary screening assay demonstrated that the pegylated material retained 45% activity with 1-3 groups of 5 kD PEG attached. A higher molar surplus

of PEG was needed to PEGylate variants in which one or several lysines were substituted with other amino acid residues.

Pegylated material was separated from unpegylated material and surplus of PEG using either size-exclusion chromatography or cation exchange chromatography or a combination of both. Size-exclusion chromatography was performed with a Superose 12 or Superdex 75 column from Pharmacia equilibrated with PBS buffer, pH 7.2. Cation exchange chromatography was performed on SP-Sepharose HP (Pharmacia) equilibrated with 20 mM citrate, pH 2.7. Elution from the SP-Sepharose HP column was performed either by increasing the concentration of salt (e.g. sodium chloride) or by increasing the pH of the buffer (e.g. sodium acetate or sodium phosphate).

### Example 12

#### *Glycosylated IFN- $\beta$ variants stabilised by the C17S substitution*

CHOK1 cells were transfected with plasmids encoding two glycosylated IFNB variants: [S2N+N4T+Q51N+E53T]hIFN- $\beta$  (PF276) and [S2N+N4T+C17S+Q51N+E53T]hIFN- $\beta$  (PF279). Confluent stable primary transfection pools were expanded into four T-175 flasks each. At confluency, the flasks were shifted from serum containing medium to a serum-free medium based on DMEM/F-12 medium (Lifetechnologies #21045-025) supplemented with 1/100 ITSA (Life Technologies #51300-044) and 1/1000 Ex-Cyte (Serologicals Corp. #81-129). Every day, in 15 days, 120 ml of each variant was harvested and frozen at  $-80^{\circ}\text{C}$ .

The supernatants from the daily harvest were collected and filtered through 0.22  $\mu\text{m}$  filter (PVDF based). The supernatant was concentrated approximately 15 times on a Vivaflow 200 system equipped with a polyethersulfon membrane with cut-off 10000 and the concentrated sample was applied on a S-Sepharose column equilibrated with 50 mM sodium acetate, 50 mM NaCl, pH 5.5. The IFNB variant eluted in a step with 50 mM sodium acetate, 0.5 M NaCl, pH 5.5.

The concentration of sodium chloride in the eluate from the S-Sepharose column was adjusted to 1.0 M and the sample was applied on a Phenyl-Sepharose column equilibrated with 50 mM sodium acetate, 1.0 M sodium chloride, pH 5.5. Extensive washing with the equilibration buffer was carried out before the IFNB variant was eluted with 60% ethylene glycol in 50 mM sodium acetate, pH 5.5.

Unreduced SDS-PAGE following the purification clearly demonstrated the formation of dimer in case of the [S2N+N4T+Q51N+E53T]hIFN- $\beta$  variant, whereas no dimer was present in case of [S2N+N4T+C17S+Q51N+E53T]hIFN- $\beta$ .

### Example 13

#### *Production, purification, and PEGylation of the [C17S+Q49N+Q51T+D110F+F111N+R113T]hIFN- $\beta$ variant*

A CHOK1 sub-clone (5/G-10) producing the [C17S+Q49N+Q51T+D110F+F111N+R113T]hIFN- $\beta$  variant was seeded into 6 roller bottles, each with an expanded surface of 1700 cm<sup>2</sup> (Corning, USA), in 200 ml DMEM/F-12 medium (Life Technologies; Cat. # 31330) supplemented with 10% FBS and penicillin/streptomycin (P/S). After 2 days the medium was exchanged. After another 2 days the two roller bottles were nearly 100% confluent and the medium was shifted to 300 ml serum-free UltraCHO medium (BioWhittaker; Cat. # 12-724) supplemented with 1/500 EX-CYTE (Serologicals Proteins; Cat. # 81129N) and P/S. Growing the cells in this medium promotes a higher cell mass, higher than can be achieved in the serum containing medium. After 2 days the medium was renewed. After another 2 days the medium was shifted to the production medium: DMEM/F-12 medium (Life Technologies; Cat. # 21041) supplemented with 1/100 ITSA (Life Technologies; Cat. # 51300-044) [ITSA stands for Insulin (1.0 g/L) – Transferrin (0.55 g/L) – Selenium (0.67 mg/L) supplement for Adherent cultures], 1/500 EC-CYTE and P/S. The harvested media from the roller bottles were pooled before a medium sample was taken out for IFNB activity determination. Every day, in 21 days, 1.8 l medium was harvested and frozen at -80 °C.

The harvested media from roller bottles was centrifuged and filtered through a 0.22  $\mu$ m filter (PVDF). The filtrated media was diafiltrated on a Vivaflow 200 system equipped with a polyethersulfon membrane with cut off 10000 and applied to a S-Sepharose column (Pharmacia).

The S-Sepharose column was equilibrated with 50 mM sodium acetate, 50 mM sodium chloride, pH 5.5 and the interferon variant was eluted with 50 mM sodium acetate, 0.5 M sodium chloride, pH 5.5. The concentration of sodium chloride in the eluate was adjusted to 1.0 M.

The eluate from the S-Sepharose column was applied on a Phenyl-Sepharose High Performance column (Pharmacia) equilibrated with 50 mM sodium acetate, 1.0 M sodium chloride, pH 5.5. Following application the column was washed with 50 mM sodium acetate, 50

mM sodium chloride, pH 5.5. The IFNB variant was eluted with a gradient from 50 mM sodium acetate, 50 mM sodium chloride, pH 5.5 to 60% ethylene glycol, 50 mM sodium acetate, pH 5.5 in 30 column volumes. Fractions containing fully glycosylated IFNB variant were collected and pooled.

The ethylene glycol in the eluate from the Phenyl-Sepharose was removed by passing the eluate through a S-Sepharose column equilibrated with 50 mM sodium acetate, 50 mM sodium chloride, pH 5.5. The ethylene glycol was in the flow through where as the interferon variant bound to the column. Following application the column was washed with 20 mM sodium acetate, pH 5.5 and the interferon variant was eluted with 100 mM sodium phosphate, pH 7.5.

The phosphate concentration in the eluate was adjusted to 15 mM sodium phosphate buffer, pH 7.2. and applied on a hydroxyapatite column (CHT I, Ceramic hydroxyapatite, Type I, Biorad) equilibrated with 15 mM sodium phosphate, pH 7.2. The fully glycosylated form passed through the column where as the underglycosylated form with one extra site used bound to the column and was eluted with a linear sodium phosphate gradient from 15 mM to 200 mM sodium phosphate, pH 6.8 in 20 column volumes.

The purity of the fully glycosylated variant [C17S+Q49N+Q51T+D110F+F111N+R113T]hIFN- $\beta$  was judged to be higher than 95% based on SDS-PAGE.

Following purification the variant was PEGylated. A fresh stock solution of 10 mg/ml SCM-PEG (succinimidyl ester of carboxymethylated PEG from Shearwater, Alabama, 12 K or 20 K) was prepared in 96 % ethanol before each experiment.

A protein solution of 0.1 mg/ml in 20 mM sodium phosphate, pH 7.0 was PEGylated with SCM-PEG, 20K, with 0.75 times molar surplus of PEG to possible PEGylation sites, i.e. lysines and N-terminus. After incubation for 30 min at room temperature, the reaction was quenched by addition of a surplus of 20 mM glycine, pH 8.0. The reaction mixture contained a mixture of mono-, di- and un-pegylated material. Mono-pegylated material was separated from other species using either cation exchange chromatography or size-exclusion chromatography or a combination of both. pH in the PEGylation solution was adjusted to pH 2.7 and the sample was applied on a SP-Sepharose HR (Pharmacia) column equilibrated with 20 mM sodium citrate, pH 2.7. The pegylated protein was eluted from the column with 50 mM sodium acetate containing 1 M sodium chloride and applied on a size-exclusion column, Sephacryl S-100, ((16/60) Pharmacia) equilibrated with 100 mM sodium acetate, 200 mM sodium chloride, pH 5.5. Fractions containing mono-pegylated material were pooled and characterized further.

In another experiment a protein solution of 0.16 mg/ml in 20 mM sodium phosphate, pH 7.0 was PEGylated with SCM-PEG, 12K, with 2 times molar surplus of PEG to possible PEGylation sites, i.e. lysines and N-terminus. After incubation for 30 min at room temperature, the reaction was quenched by addition of a surplus of 20 mM glycine, pH 8.0. The reaction mixture contained a mixture of mono-, di-, tri-pegylated material together with underivatized material. The pegylated material was separated from the unmodified protein using either cation exchange chromatography or size-exclusion chromatography or a combination of both. pH in the PEGylation solution was adjusted to pH 2.7 and the sample was applied on a SP-Sepharose HR (Pharmacia) column equilibrated with 20 mM sodium citrate, pH 2.7. The pegylated protein was eluted from the column with 50 mM sodium acetate containing 1 M sodium chloride and applied on a size-exclusion column, Sephacryl S-100, ((16/60) Pharmacia) equilibrated with 100 mM sodium acetate, 200 mM sodium chloride, pH 5.5. Fractions containing the mixture of mono-, di- and tri-pegylated protein were pooled and characterized further.

#### **Example 14**

##### *Production, purification, and PEGylation of the [C17S+K19R+K33R+K45R+Q49N+Q51T+D110F+F111N+R113T]hIFN- $\beta$ variant*

A CHOK1 sub-clone (5/G-10) producing the [C17S+K19R+K33R+K45R+Q49N+Q51T+D110F+F111N+R113T]hIFN- $\beta$  glycosylation variant was produced in 6 roller bottles as described in Example 13 and purified according to the protocol used in Example 13. The purity of the fully glycosylated variant [C17S+K19R+K33R+K45R+Q49N+Q51T+D110F+F111N+R113T]IFNB was judged to be higher than 95% based on SDS-PAGE.

Following purification the variant was PEGylated. A fresh stock solution of SCM-PEG (succinimidyl ester of carboxymethylated PEG from Shearwater, Alabama, 12 kD or 20 kD) was prepared in ethanol before each experiment.

A protein solution of 0.1 mg/ml in 20 mM sodium phosphate, pH 7.0 was PEGylated with SCM-PEG, 20K, with 3 times molar surplus of PEG to possible PEGylation sites, i.e. lysines and N-terminus. After incubation for 30 min at room temperature, the reaction was quenched by addition of a surplus of 20 mM glycine, pH 8.0. The reaction mixture contained a mixture of mono-, di- and un-pegylated material. Mono-pegylated material was separated from other species using either cation exchange chromatography or size-exclusion chromatography or a combination of both. pH in the PEGylation solution was adjusted to pH 2.7 and the sample was applied on a SP-Sepharose HR (Pharmacia) column equilibrated with 20 mM sodium

citrate, pH 2.7. The pegylated protein was eluted from the column with 50 mM sodium acetate containing 1 M sodium chloride and applied on a size-exclusion column, Sephacryl S-100, ((16/60) Pharmacia) equilibrated with 100 mM sodium acetate, 200 mM sodium chloride, pH 5.5. Fractions containing mono-pegylated material was pooled and characterized further

In another experiment a protein solution of 0.1 mg/ml in 20 mM sodium phosphate, pH 7.0 was PEGylated with (10 mg/ml) SCM-PEG, 12K, with 5 times molar surplus of PEG to possible PEGylation sites, i.e. lysines and N-terminus. After incubation for 30 min at room temperature, the reaction was quenched by addition of a surplus of 20 mM glycine, pH 8.0. The reaction mixture contained a mixture of mono-, di-, tri-pegylated material together with underivatized material. The pegylated material was separated from the unmodified protein using either cation exchange chromatography or size-exclusion chromatography or a combination of both. pH in the PEGylation solution was adjusted to pH 2.7 and the sample was applied on a SP-Sephacryl HR (Pharmacia) column equilibrated with 20 mM sodium citrate, pH 2.7. The pegylated protein was eluted from the column with 50 mM sodium acetate containing 1 M sodium chloride and applied on a size-exclusion column, Sephacryl S-100, ((16/60) Pharmacia) equilibrated with 100 mM sodium acetate, 200 mM sodium chloride, pH 5.5. Fractions containing the mixture of mono-, di- and tri-pegylated protein were pooled and characterized further.

#### Example 14

##### *Increased in vivo half-life*

Pharmacokinetics of the following variants:

- i) 12 kDa mono-PEG [C17S+Q49N+Q51T+D110F+F111N+R113T]hIFN- $\beta$  (**variant #1**),
- ii) 20 kDa mono-PEG [C17S+Q49N+Q51T+D110F+F111N+R113T]hIFN- $\beta$  (**variant #2**),
- iii) 12 kDa mono-PEG [C17S+K19R+K33R+K45R+Q49N+Q51T+D110F+F111N+R113T]hIFN- $\beta$  (**variant #3**), and
- iv) 20 kDa mono-PEG [C17S+K19R+K33R+K45R+Q49N+Q51T+D110F+F111N+R113T]hIFN- $\beta$  (**variant #4**) as well as Rebif® were assessed in minipigs after subcutaneous and intravenous administration. Three animals within each group were tested. 600,000 U/kg, 0.05 ml/kg) was administered in all experiments. The mean half-lives shown in Table 1 were obtained:

Compound	Route	T <sub>1/2</sub> (hrs)	PEG size kDa
Variant #1	iv	30	12
Variant #1	sc	41	12
Variant #2	iv	38	20
Variant #2	sc	38	20
Variant #3	iv	34	12
Variant #3	sc	32	12
Variant #4	iv	34	20
Variant #4	sc	26	20
Rebif®	iv	16	-
Rebif®	sc	3	-

Table 1

**Example 15**

*Anti-tumorogenic activity of 20kDa mono-pegylated [C17S+Q49N+Q51T+D110F+F111N+R113T]hIFN- $\beta$  variant (prepared in Example 13)*

*Materials and methods*

The variant in a low and high dosis, and for comparison Rebif® at a high dosis, were evaluated for the potential inhibitory effect on the growth of a malignant human melanoma (A375.S2) grown as xenograft in nude (*nu/nu*) mice.

The experiments were performed in a total of 90 six-week-old female Bom: NMRI-nu® (M&B, Ry, Denmark) athymic nude mice, two series of 45 mice each. An acclimatization period of one week was allowed in order to exclude animals in poor condition.

The mice were kept under sterile conditions in individually sterile-air ventilated Sealsafe® cage racks (Scanbur A/S, Lelling, Denmark). Sealsafe® macrolon cages with 2-5 animals in each cage was used. The mice were allowed sterile water and food pellets *ad libitum*. The cages and the bedding were changes once a week. The room temperature was 25°C  $\pm$  2°C and the relative humidity was 55%  $\pm$  5%. The room was illuminated 24 hours a day.

The experiments were performed on the A375.S2 human malignant melanoma (ATTC) which were kept as serially transplanted tumor xenografts *in vivo* in nude mice. After acclimatization, approximately 2-mm-diameter tumor blocks from serially transplanted A375.S2 tumors were inoculated subcutaneously in both flanks of the recipient mice. Tumor transplantations were performed in sterile laminar air-flow benches and under general anaesthesia with Rompun® and Ketalar®. Each animal was identified by earmarks and each cage was marked to identify earmarked animals.

During the experimental period the mice were weighed once a week, and the mice were observed daily for possible side-effects.

When the tumors became measurable, they were measured daily in two dimensions ( $d_1$  and  $d_2$ ) using a slide gauche. The tumor measurements were used to monitor the tumor growth during the experiment. Calculated tumor areas ( $d_1$  and  $d_2$ ) were used to construct growth curves according to the transformed Gompertz function:

$$\ln[\ln A(\max) - \ln A(t)] = \ln (\beta/\alpha) - \alpha t$$

This function depicts the growth as a straight line when the tumor size  $\ln[\ln A(\max) - \ln A(t)]$  is plotted against time ( $t$ ).  $A(\max)$  is the theoretical maximum tumor area,  $A(t)$  is the tumor area at time  $t$ , and  $\alpha$  and  $\beta$  are constants. The negative slope ( $\alpha$ ) of the growth curve represents the specific growth rate of the tumor.

The transformed Gompertz function was also used to construct normalized mean tumor area and mean volume growth curves after termination of the experiment and to calculate growth curve characteristics.

Tumor volume,  $V(t)$ , at time  $t$  was calculated from the tumor area,  $A(t)$ , according to the formula:

$$V(t) = \pi/6 A(t)^{1.5} k,$$

where  $k$  ( $= 0.67$ ) is a constant defining the relation between the three perpendicular dimensions of the tumor.

The tumor volume doubling time,  $TD$ , was calculated according to the formula:

$$TD = -1/\alpha \ln[1 + \ln 2/(\ln V(t)/\ln V(\max))]$$



Effective treatment will induce cell kill, and the tumors will re-grow with a treatment-induced delay. The effect of the treatment was determined from the normalized mean transformed Gompertz growth curves by calculation of the specific growth delay, SGD, according to the formula:

$$\text{SGD} = (\text{TGD} - \text{CGD})/\text{CGD},$$

Where TGD is the growth delay of the tumors in a group of treated tumors, and CGD is the growth delay of the control group. Since the time to reach twice the volume at the time of the first day of treatment was used, the calculated SGD values are the treatment-induced number of spared doubling times.

Tumors were excluded from the experiment according to the following criteria: *i*) tumors without sufficient growth for establishment of a pretreatment growth curve at the time of randomization, *ii*) tumors exceeding or close to the maximum permitted size at the time of randomization, and *iii*) tumors in mice dying before termination of the experiment.

Pretreatment growth curves based on at least six consecutive measurements were established. When the pretreatment growth curves were established the tumors were randomized into four treatment groups. The mice in each group were treated two times with 0.1 ml s.c. of one of the four blinded test substances (vehicle (control), Rebif® 1.2 MIU/ml, variant 0.25 MIU/ml, variant 1.5 MIU/ml). After possible treatment-induced response, tumor measurements were used to establish unperturbed posttreatment regrowth curves based on at least six consecutive measurements. When posttreatment growth curves of a treatment group were established the experiment was terminated for this group by sacrificing the mice by cervical dislocation.

After termination of all four groups, normalized mean transformed Gompertz growth curves were constructed and used for calculation of treatment-induced SGD. The code of the blinded test substances was not broken until after calculation of the treatment effect of the treatment groups. The mice were weighed once weekly. After termination of each experimental group, tumor specimens were prepared for histological and immunohistochemical examination.

At day 15 after transplantation, the tumors were randomized into four treatment groups. Tumors demonstrating growth during (at least) six consecutive measurements were included. The experiment included 49 tumors in 34 mice.

A total of 41 of the 90 transplanted tumors had to be excluded according to the employed criteria mentioned above.

The blinded treatment substances were labeled Test A, B, C and D. Each group of mice were treated with one of the test substances, 0.1 ml s.c., two times a week for two weeks.

### Results

None of the included 34 mice died during the experiment, and the daily observation of the animals revealed no signs of wasting or other side-effects. Furthermore, the results of the weighing of the animals revealed that the treatment did not affect the weight of the animals. Thus, it can be concluded that the employed doses of the drugs were non-toxic to the animals.

The daily measurements were used to calculate tumor area values and to construct rectilinear transformed Gompertz growth curves of the individual tumors. The calculated Gompertz growth data were used to construct mean normalized growth curves. The corresponding mean normalized tumor volume growth curve is shown in Fig. 1.

The specific growth delay was calculated by determining the time to grow twice the mean tumor volume at initiation of treatment in the control and treated groups of tumors. The obtained SGD-values are shown in Table 2 below together with the other calculated growth curve characteristics.

Group	Number		$\alpha$	$\beta$	$r$	TD	GD	SGD
	mice	tumors	$\times 10^{-2}$	$\times 10^{-1}$		days	days	
Control	8	12	8.2	7.6	0.982	2.6	2.3	0.0
Rebif®	9	13	7.3	6.6	0.996	3.0	4.2	0.8
Variant(low)	8	13	6.2	5.6	0.989	3.5	7.6	2.3
Variant(high)	7	9	6.0	15.3	0.977	3.6	25.7	10.2

Table 2

With the employed dose, Rebif® had a small but significant growth inhibitory effect. The treatment with the variant resulted in a dose-dependent effect. Both doses of the variant had greater growth inhibitory effect than Rebif®, and the SGD-value of 10.2 following high-dose treatment is extremely high and is rarely seen in experimental cancer therapy. In addition, the SGD-value of 10.2 is an underestimation of the effect, since only nine of the eleven treated tumors were included in the SGD-calculation, because two of the treated tumors (18%) of this

treatment group were "cured", i.e. they did not show regrowth during the observation period. The SGD-value of the cured tumors is infinite. Thus, the true value following high-dose treatment with the variant was between 10.2 and  $\infty$ .

In a similar study, the variant was compared to Pegasys® (Pegylated interferon  $\alpha$ -2a). It was found that the SGD value for the variant (6 MIU/kg body weight, corresponding to 90  $\mu$ g/kg body weight) was improved 5- to 7-fold as compared to Pegasys® (6 MIU/kg body weight, corresponding to 90  $\mu$ g/kg body weight).

**CLAIMS**

1. Use of an interferon  $\beta$  (IFNB) polypeptide with an increased functional *in vivo* half-life as compared to interferon  $\beta$ 1a for the manufacture of a medicament for the treatment of a cancer having malignant cells carrying interferon type 1 deletions.
2. Use according to claim 1, wherein said malignant cells carrying interferon type 1 deletions have a deletion of the short arm of chromosome 9 (9p22).
3. Use according to claim 1 or 2, wherein said cancer is a carcinoma.
4. Use according to claim 3, wherein said carcinoma is selected from the group consisting of malignant melanoma, basal cell carcinoma, ovarian carcinoma, breast carcinoma, non-small cell lung cancer, renal cell carcinoma, bladder carcinoma, recurrent superficial bladder cancer, stomach carcinoma, prostatic carcinoma, pancreatic carcinoma, lung carcinoma, cervical carcinoma, cervical dysplasia, laryngeal papillomatosis, colon carcinoma, colorectal carcinoma and carcinoid tumors.
5. Use according to claim 4, wherein said carcinoma is selected from the group consisting of malignant melanoma, non-small cell lung cancer, breast carcinoma, colon carcinoma and renal cell carcinoma.
6. Use according to claim 5, wherein said malignant melanoma is selected from the group consisting of superficial spreading melanoma, nodular melanoma, lentigo maligna melanoma, acral melanoma, amelanotic melanoma and desmoplastic melanoma.
7. Use according to claim 1 or 2, wherein said cancer is a sarcoma.
8. Use according to claim 7, wherein said sarcoma is selected from the group consisting of osteosarcoma, Ewing's sarcoma, chondrosarcoma, malignant fibrous histiocytoma, fibrosarcoma and Kaposi's sarcoma.
9. Use according to claim 1 or 2, wherein said cancer is a glioma.

10. Use according to any of the preceding claims, wherein said cancer is in the form of a solid malignant tumor.
11. Use according to claim 10, wherein said medicament is administered intratumorally.
12. Use according to claim 11, wherein said medicament is administered intratumorally by direct injection into the tumor.
13. Use according to claim 1 or 2, wherein said cancer is a leukaemia.
14. Use according to claim 13, wherein said leukaemia is selected from the group consisting of acute myelogenous leukaemia, chronic myelogenous leukaemia, acute lymphocytic leukaemia, chronic lymphocytic leukaemia and hairy cell leukaemia.
15. Use according to claim 1 or 2, wherein said cancer is a lymphoma.
16. Use according to claim 15, wherein said lymphoma is selected from the group consisting of Hodgkin's disease, non-Hodgkin's disease, B-cell lymphoma, T-cell lymphoma, follicular lymphoma, Burkitt's lymphoma and mycosis fungoides.
17. Use according to claim 1 or 2, wherein said cancer is a myeloma.
18. Use according to claim 17, wherein said myeloma is multiple myeloma.
19. Use according to any of the preceding claims, wherein said IFNB polypeptide is used in combination with a chemotherapeutic agent.
20. Use according to claim 19, wherein the chemotherapeutic agent is selected from the group consisting of adrenocorticosteroids, such as prednisone, dexamethasone or decadron; altretamine (hexalen, hexamethylmelamine (HMM)); amifostine (ethyol); aminoglutethimide (cytadren); amsacrine (M-AMSA); anastrozole (arimidex); androgens, such as testosterone; asparaginase (elspar); bacillus calmette-gurin; bicalutamide (casodex); bleomycin (blenoxane);

busulfan (myleran); carboplatin (paraplatin); carmustine (BCNU, BiCNU); chlorambucil (leukeran); chlorodeoxyadenosine (2-CDA, cladribine, leustatin); cisplatin (platinol); cytosine arabinoside (cytarabine); dacarbazine (DTIC); dactinomycin (actinomycin-D, cosmegen); daunorubicin (cerubidine); docetaxel (taxotere); doxorubicin (adriamycin); epirubicin; estramustine (emcyt); estrogens, such as diethylstilbestrol (DES); etoposide (VP-16, VePesid, etopophos); fludarabine (fludara); flutamide (eulexin); 5-FUDR (floxuridine); 5-fluorouracil (5-FU); gemcitabine (gemzar); goserelin (zodalex); herceptin (trastuzumab); hydroxyurea (hydrea); idarubicin (idamycin); ifosfamide; IL-2 (proleukin, aldesleukin); interferon alpha (intron A, roferon A); irinotecan (camptosar); leuprolide (lupron); levamisole (ergamisole); lomustine (CCNU); mechlorathamine (mustargen, nitrogen mustard); melphalan (alkeran); mercaptopurine (purinethol, 6-MP); methotrexate (mexate); mitomycin-C (mutamycin); mitoxantrone (novantrone); octreotide (sandostatin); pentostatin (2-deoxycoformycin, nipent); plicamycin (mithramycin, mithracin); prorocarbazine (matulane); streptozocin; tamoxifen (nolvadex); taxol (paclitaxel); teniposide (vumon, VM-26); thiotepa; topotecan (hycamtin); tretinoin (vesanoid, all-trans retinoic acid); vinblastine (valban); vincristine (oncovin) and vinorelbine (navelbine).

21. Use according to claim 20, wherein said cancer and said chemotherapeutic agent is selected from the group consisting of malignant melanoma and cisplatin; malignant melanoma and IL-2; renal cell carcinoma and doxorubicin; renal cell carcinoma and IL-2; breast carcinoma and doxorubicin; breast carcinoma and taxol; colon carcinoma and 5-fluorouracil; colon carcinoma and cisplatin; and non-small cell lung cancer and cisplatin.

22. Use according to any of the preceding claims, wherein said IFNB polypeptide is used in combination with radiotherapy.

23. Use according to any of claims 1-22, wherein said IFNB polypeptide is an IFNB variant comprising an amino acid sequence which differs from the amino acid sequence of wild-type human IFNB (SEQ ID NO 2) in 1-15 amino acid residues.

24. Use according to claim 23, wherein said variant comprises at least one introduced glycosylation site.

25. Use according to claim 24, wherein a sugar moiety is covalently attached to said introduced glycosylation site

26. Use according to claim 24 or 25, wherein said glycosylation site is an *in vivo* N-glycosylation site.

27. Use according to claim 26, wherein said at least one glycosylation site is introduced by a substitution selected from the group consisting of S2N+N4T/S, L9N+R11T/S, R11N, S12N+N14T/S, F15N+C17S/T, Q16N+Q18T/S, K19N+L21T/S, Q23N+H25T/S, G26N+L28T/S, R27N+E29T/S, L28N+Y30T/S, D39T/S, K45N+L47T/S, Q46N+Q48T/S, Q48N+F50T/S, Q49N+Q51T/S, Q51N+E53T/S, R71N+D73T/S, Q72N, D73N, S75N, S76N+G78T/S, L88T/S, Y92T/S, N93N+I95T/S, L98T/S, E103N+K105T/S, E104N+L106T/S, E107N+E109T/S, K108N+D110T/S, D110N, F111N+R113T/S and L116N.

28. Use according to claim 27, wherein said substitutions are selected from the group consisting of S2N+N4T, L9N+R11T, Q49N+Q51T, R71N+D73T and F111N+R113T.

29. Use according to claim 28, wherein said substitutions are selected from the group consisting of Q49N+Q51T, R71N+D73T and F111N+R113T.

30. Use according to claim 29, wherein said substitutions are selected from the group consisting of Q49N+Q51T and F111N+R113T.

31. Use according to any of claims 24-30, wherein said variant comprises at least two introduced glycosylation sites.

32. Use according to claim 31, wherein said variant comprises two introduced glycosylation sites.

33. Use according to claim 31 or 32, wherein said glycosylation sites are *in vivo* N-glycosylation sites.

34. Use according to claim 33, wherein said variant comprises substitutions selected from the group consisting of

Q49N+Q51T+F111N+R113T, Q49N+Q51T+R71N+D73T+ F111N+ R113T,  
S2N+N4T+F111N+R113T, S2N+N4T+Q49N+Q51T,  
S2N+N4T+Q49N+Q51T+F111N+R113T, S2N+N4T+L9N+R11T+Q49N+Q51T,  
S2N+N4T+L9N+R11T+F111N+R113T,  
S2N+N4T+L9N+R11T+Q49N+Q51T+F111N+R113T, L9N+R11T+Q49N+Q51T,  
L9N+R11T+Q49N+Q51T+F111N+R113T and L9N+R11T+F111N+R113T.

35. Use according to claim 34, wherein said variant comprises the substitutions  
Q49N+Q51T+F111N+R113T.

36. Use according to any of claims 23-35, wherein the cysteine residue located at position 17 in human wild-type IFNB (SEQ ID NO 2) has been removed.

37. Use according to claim 36, wherein said cysteine residue has been removed by the substitution C17S.

38. Use according to claim 37, wherein said variant comprises substitutions selected from the group consisting of S2N+N4T/S+C17S, L9N+R11T/S+C17S, R11N+C17S,  
S12N+N14T/S+C17S, F15N+C17S, Q16N+C17S+Q18T/S, C17S+K19N+L21T/S,  
C17S+Q23N+H25T/S, C17S+G26N+L28T/S, C17S+R27N+E29T/S, C17S+L28N+Y30T/S,  
C17S+D39T/S, C17S+K45N+L47T/S, C17S+Q46N+Q48T/S, C17S+Q48N+F50T/S,  
C17S+Q49N+Q51T/S, C17S+Q51N+E53T/S, C17S+R71N+D73T/S, C17S+Q72N,  
C17S+D73N, C17S+S75N, C17S+S76N+G78T/S, C17S+L88T/S, C17S+Y92T/S,  
C17S+N93N+I95T/S, C17S+L98T/S, C17S+E103N+K105T/S, C17S+E104N+L106T/S,  
C17S+E107N+E109T/S, C17S+K108N+D110T/S, C17S+D110N, C17S+F111N+R113T/S and  
C17S+L116N.

39. Use according to claim 38, wherein said variant comprises substitutions selected from the group consisting of S2N+N4T+C17S, L9N+R11T+C17S, C17S+Q49N+Q51T,  
C17S+F111N+R113T, C17S+Q49N+Q51T+F111N+R113T,  
C17S+Q49N+Q51T+R71N+D73T+ F111N+R113T, S2N+N4T+C17S+F111N+R113T,



S2N+N4T+C17S+Q49N+Q51T, S2N+N4T+C17S+Q49N+Q51T+F111N+R113T,  
S2N+N4T+L9N+R11T+C17S+Q49N+Q51T, S2N+N4T+L9N+R11T+C17S+F111N+R113T,  
S2N+N4T+L9N+R11T+C17S+Q49N+Q51T+F111N+R113T,  
L9N+R11T+C17S+Q49N+Q51T, L9N+R11T+C17S+Q49N+Q51T+F111N+R113T and  
L9N+R11T+C17S+F111N+R113T.

40. Use according to claim 39, wherein said variant comprises the substitutions  
C17S+Q49N+Q51T+F111N+R113T.

41. Use according to any of claims 23-40, wherein said variant comprises a substitution in  
position 110.

42. Use according to claim 41, wherein said substitution is selected from the group consisting  
of D110F, D110V, D110W and D110Y.

43. Use according to claim 42, wherein said substitution is D110F.

44. Use according to claim 43, wherein said variant comprises substitutions selected from the  
group consisting of

D110F+F111N+R113T, Q49N+Q51T+D110F+F111N+R113T,  
Q49N+Q51T+R71N+D73T+D110F+F111N+R113T, S2N+N4T+D110F+F111N+R113T,  
S2N+N4T+Q49N+Q51T+D110F+F111N+R113T,  
S2N+N4T+L9N+R11T+D110F+F111N+R113T,  
S2N+N4T+L9N+R11T+Q49N+Q51T+D110F+F111N+R113T,  
L9N+R11T+Q49N+Q51T+D110F+F111N+R113T and L9N+R11T+D110F+F111N+R113T.

45. Use according to claim 44, wherein said variant comprises the substitutions  
Q49N+Q51T+D110F+F111N+R113T.

46. Use according to any of claims 43-45, wherein said variant comprises substitutions selected  
from the group consisting of

C17S+D110F+F111N+R113T, C17S+Q49N+Q51T+D110F+F111N+R113T,  
C17S+Q49N+Q51T+R71N+D73T+D110F+F111N+R113T,

S2N+N4T+C17S+D110F+F111N+R113T,  
S2N+N4T+C17S+Q49N+Q51T+D110F+F111N+R113T,  
S2N+N4T+L9N+R11T+C17S+D110F+F111N+R113T,  
S2N+N4T+L9N+R11T+C17S+Q49N+Q51T+D110F+F111N+R113T,  
L9N+R11T+C17S+Q49N+Q51T+D110F+F111N+R113T and  
L9N+R11T+C17S+D110F+F111N+R113T.

47. Use according to claim 46, wherein said variant comprises the substitutions  
C17S+Q49N+Q51T+D110F+F111N+R113T.

48. Use according to claim 47, wherein said variant has the amino acid sequence shown in SEQ  
ID NO 3.

49. Use according to any of claims 23-48, wherein a polymer molecule is covalently attached to  
an amino acid residue of the IFNB polypeptide.

50. Use according to claim 49, wherein no glycosylation sites have been introduced.

51. Use according to claim 50, wherein said IFNB polypeptide comprises at least one  
introduced glycosylation site.

52. Use according to claim 51, wherein said glycosylation site is as defined in any of claims 25-  
48.

53. Use according to any of claims 1-22, wherein said IFNB polypeptide has the amino acid  
sequence shown in SEQ ID NO 2 and wherein a polymer molecule is covalently attached to an  
amino acid residue of the IFNB polypeptide.

54. Use according to any of claims 49-53, wherein said polymer is a PEG molecule.

55. Use according to any of claims 49-54, wherein said attachment group is the  $\epsilon$ -amino group  
of a lysine residue or the N-terminal amino group.

56. Use according to any of claims 49-52 or 54-55, wherein at least one lysine residue has been removed.

57. Use according to claim 56, wherein said lysine residue is selected from the group consisting of K19, K33, K45, K52, K99, K105, K108, K115, K123, K134 and K136.

58. Use according to claim 57, wherein said lysine residue is selected from the group consisting of K19, K33, K45 and K123.

59. Use according to any of claims 56-58, wherein said lysine residue has been removed by substituting said lysine residue with an arginine or glutamine residue.

60. Use according to claim 59, wherein said substitution(s) is (are) selected from the group consisting of K19R, K33R, K45R, K123R, K19R+K33R, K19R+K45R, K19R+K123R, K33R+K45R, K33R+K123R, K45R+K123R, K19R+K45R+K123R, K19R+K33R+K123R, K19R+K33R+K45R, K33R+K45R+K123R and K19R+K33R+K45R+K123R.

61. Use according to claim 60, wherein said substitutions are selected from the group consisting of K19R+K45R+K123R, K19R+K33R+K123R, K19R+K33R+K45R and K33R+K45R+K123R.

62. Use according to claim 61, wherein said substitutions are K19R+K33R+K45R.

63. Use according to claim 62, wherein said variant comprises substitutions selected from the group consisting of

C17S+Q49N+Q51T+K19R+K33R+K45R,

C17S+D110F+F111N+R113T+K19R+K33R+K45R,

C17S+Q49N+Q51T+D110F+F111N+R113T+K19R+K33R+K45R,

C17S+Q49N+Q51T+R71N+D73T+D110F+F111N+ R113T+K19R+K33R+K45R,

S2N+N4T+C17S+D110F+F111N+R113T+K19R+K33R+K45R,

S2N+N4T+C17S+Q49N+Q51T+D110F+F111N+R113T+K19R+K33R+K45R,

S2N+N4T+L9N+R11T+C17S+D110F+F111N+R113T+K19R+K33R+K45R,

S2N+N4T+L9N+R11T+C17S+Q49N+Q51T+D110F+F111N+R113T+K19R+K33R+K45R,

L9N+R11T+C17S+Q49N+Q51T+D110F+F111N+R113T+K19R+K33R+K45R and  
L9N+R11T+C17S+D110F+F111N+R113T+K19R+K33R+K45R.

64. Use according to claim 63, wherein said variant comprises the substitutions  
C17S+Q49N+Q51T+D110F+F111N+R113T+K19R+K33R+K45R.

65. Use according to claim 64, wherein said variant has the amino acid sequence shown in SEQ  
ID NO 4.

66. Use according to any of claims 54-65, wherein said PEG molecule has a molecular weight  
of about 12 kDa or about 20 kDa.

67. Use according to claim 66, wherein said PEG molecule has a molecular weight of about 20  
kDa.

68. Use according to any of claims 54-67, wherein said IFNB polypeptide comprises a single  
PEG molecule.

69. Use of an interferon  $\beta$  (IFNB) polypeptide with an increased functional *in vivo* half-life as  
compared to interferon  $\beta$ 1a in combination with a chemotherapeutic agent for the manufacture  
of a medicament for the treatment of cancer.

70. Use according to claim 69, wherein said cancer is as defined in any of claims 3-10 or 13-18.

71. Use according to claim 69 or 70, wherein said medicament is administered as defined in  
claims 11 or 12.

72. Use according to any of claims 69-71, wherein said chemotherapeutic agent is as defined in  
claims 20 or 21.

73. Use according to any of claims 69-72, wherein said medicament is used in combination  
with radiotherapy.

74. Use according to any of claims 69-73, wherein said IFNB polypeptide is as defined in any of claims 23-68.

75. A method of treating a mammal having a cancer, wherein said cancer has malignant cells carrying interferon type 1 deletions, said method comprising administering to said mammal a therapeutically effective dose of an interferon beta (IFNB) polypeptide with increased functional *in vivo* half-life as compared to interferon  $\beta$ 1a.

76. A method of treating a mammal having cancer, said method comprising administering to said mammal a therapeutically effective dose of an interferon beta (IFNB) polypeptide with increased functional *in vivo* half-life as compared to interferon  $\beta$ 1a in combination with a chemotherapeutic agent.

77. The method according to claim 75 or 76, wherein said mammal is a human being.

78. A pharmaceutical composition comprising

- i) an interferon beta (IFNB) polypeptide with increased functional *in vivo* half-life as compared to interferon  $\beta$ 1a;
- ii) a chemotherapeutic agent; and
- iii) a pharmaceutically acceptable diluent, carrier or adjuvant.

79. The composition according to claim 78, wherein said chemotherapeutic agent is as defined in claims 20 or 21.

80. The composition according to claim 78 or 79, wherein said IFNB polypeptide is as defined in any of claims 23-68.

1/1

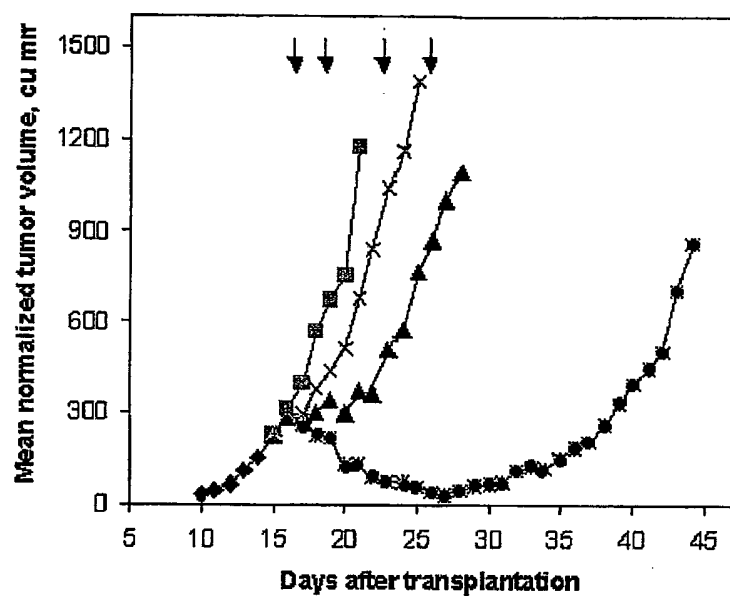


Fig. 1

## SEQUENCE LISTING

&lt;110&gt; Maxygen ApS

&lt;120&gt; Interferon beta-like molecules for treatment of cancer

&lt;130&gt; 0263wo210 - INFB for cancer

&lt;140&gt;

&lt;141&gt;

&lt;160&gt; 52

&lt;170&gt; PatentIn Ver. 2.1

&lt;210&gt; 1

&lt;211&gt; 840

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

<223> Description of Artificial Sequence: Expression  
cassette for expression of IFNB in mammalian  
and insect cells

&lt;400&gt; 1

```

acatttctaac tgcaaccttt cgaagccttt gctctggcac aacaggtagt aggcgacact 60
gttcgtgttg tcaacatgac caacaagtgt ctctccaaa ttgctctcct gttgtgcttc 120
tccactacag ctctttccat gagctacaac ttgcttggat tcctacaaag aagcagcaat 180
tttcagtgtc agaagctcct gtggcaattg aatgggaggc ttgaatactg cctcaaggac 240
aggatgaact ttgacatccc tgaggagatt aagcagctgc agcagttcca gaaggaggac 300
gccgcattga ccatctatga gatgctccag aacatctttg ctattttcag acaagattca 360
tctagcactg gctggaatga gactattgtt gagaacctcc tggctaattg ctatcatcag 420
ataaaccatc tgaagacagt cctggaagaa aaactggaga aagaagattt caccagggga 480
aaactcatga gcagtctgca cctgaaaaga tattatggga ggattctgca ttacctgaag 540
gcccaaggag acagtcactg tgcctggacc atagtcagag tggaaatcct aaggaacttt 600
tacttcatta acagacttac aggttacctc cgaaactgaa gatctcctag cctgtgcctc 660
tgggactgga caattgcttc aagcattcct caaccagcag atgctgttta agtgactgat 720
ggctaattga ctgcatatga aaggacacta gaagattttg aaatttttat taaattatga 780
gttattttta tttattttaa ttttattttg gaaaataaat tatttttggg gcaaaagtca 840

```

&lt;210&gt; 2

&lt;211&gt; 166

&lt;212&gt; PRT

&lt;213&gt; Homo sapiens

&lt;400&gt; 2

```

Met Ser Tyr Asn Leu Leu Gly Phe Leu Gln Arg Ser Ser Asn Phe Gln
  1                      5                      10                      15

```

```

Cys Gln Lys Leu Leu Trp Gln Leu Asn Gly Arg Leu Glu Tyr Cys Leu
      20                      25                      30

```

```

Lys Asp Arg Met Asn Phe Asp Ile Pro Glu Glu Ile Lys Gln Leu Gln
      35                      40                      45

```

```

Gln Phe Gln Lys Glu Asp Ala Ala Leu Thr Ile Tyr Glu Met Leu Gln
      50                      55                      60

```

```

Asn Ile Phe Ala Ile Phe Arg Gln Asp Ser Ser Ser Thr Gly Trp Asn

```

```
<210> 3
<211> 166
<212> PRT
<213> Artificial Sequence
```

```

<400> 3
Met Ser Tyr Asn Leu Leu Gly Phe Leu Gln Arg Ser Ser Asn Phe Gln
  1          5          10          15

Ser Gln Lys Leu Leu Trp Gln Leu Asn Gly Arg Leu Glu Tyr Cys Leu
          20          25          30

Lys Asp Arg Met Asn Phe Asp Ile Pro Glu Glu Ile Lys Gln Leu Gln
          35          40          45

Asn Phe Thr Lys Glu Asp Ala Ala Leu Thr Ile Tyr Glu Met Leu Gln
          50          55          60

Asn Ile Phe Ala Ile Phe Arg Gln Asp Ser Ser Ser Thr Gly Trp Asn
  65          70          75          80

Glu Thr Ile Val Glu Asn Leu Leu Ala Asn Val Tyr His Gln Ile Asn
          85          90          95

His Leu Lys Thr Val Leu Glu Glu Lys Leu Glu Lys Glu Phe Asn Thr
          100          105          110

Thr Gly Lys Leu Met Ser Ser Leu His Leu Lys Arg Tyr Tyr Gly Arg
          115          120          125

Ile Leu His Tyr Leu Lys Ala Lys Glu Tyr Ser His Cys Ala Trp Thr
          130          135          140

Ile Val Arg Val Glu Ile Leu Arg Asn Phe Tyr Phe Ile Asn Arg Leu
          145          150          155          160

Thr Gly Tyr Leu Arg Asn

```



```
<210> 4
<211> 166
<212> PRT
<213> Artificial Sequence
```

```

<400> 3
Met Ser Tyr Asn Leu Leu Gly Phe Leu Gln Arg Ser Ser Asn Phe Gln
  1          5          10          15

Ser Gln Arg Leu Leu Trp Gln Leu Asn Gly Arg Leu Glu Tyr Cys Leu
      20          25          30

Arg Asp Arg Met Asn Phe Asp Ile Pro Glu Glu Ile Arg Gln Leu Gln
      35          40          45

Asn Phe Thr Lys Glu Asp Ala Ala Leu Thr Ile Tyr Glu Met Leu Gln
  50          55          60

Asn Ile Phe Ala Ile Phe Arg Gln Asp Ser Ser Ser Thr Gly Trp Asn
  65          70          75          80

Glu Thr Ile Val Glu Asn Leu Leu Ala Asn Val Tyr His Gln Ile Asn
      85          90          95

His Leu Lys Thr Val Leu Glu Glu Lys Leu Glu Lys Glu Phe Asn Thr
      100          105          110

Thr Gly Lys Leu Met Ser Ser Leu His Leu Lys Arg Tyr Tyr Gly Arg
      115          120          125

Ile Leu His Tyr Leu Lys Ala Lys Glu Tyr Ser His Cys Ala Trp Thr
      130          135          140

Ile Val Arg Val Glu Ile Leu Arg Asn Phe Tyr Phe Ile Asn Arg Leu
  145          150          155          160

Thr Gly Tyr Leu Arg Asn
      165

```

```
<400> 5
ggctagcgtt taaacttaag cttcgccacc atgaccaaca agtgccctgct ccagatcgcc 60
ctgctcctgt                                     70
```

3

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 6

acaacctgct cggcttcctg cagaggagtt cgaacttcca gtgccagaag ctctgtggc 60  
agctgaacgg 70

<210> 7

<211> 70

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 7

gaacttcgac atcccgcgagg aaatcaagca gctgcagcag ttccagaagg aggacgccgc 60  
tctgaccatc 70

<210> 8

<211> 70

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 8

ttccgccagg actccagctc caccgggttg aacgagacca tcgtggagaa cctgctggcc 60  
aacgtgtacc 70

<210> 9

<211> 70

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 9

aggagaagct ggagaaggag gacttcaccc gcggcaagct gatgagctcc ctgcacctga 60  
agcgctacta 70

<210> 10

<211> 70

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 10

ggagtacagc cactgcgcct ggaccatcgt acgcgtggag atcctgcgca acttctactt 60  
catcaaccgc 70

<210> 11  
<211> 70  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer

<400> 11  
caccacactg gactagtgga tccttatcag ttgcgcaggt agccgggtcag gcggttgatg 60  
aagtagaagt 70

<210> 12  
<211> 70  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer

<400> 12  
aggcgcagtg gctgtactcc ttggccttca ggtagtgcag gatgcggcca tagtagcgct 60  
tcaggtgcag 70

<210> 13  
<211> 70  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer

<400> 13  
ctcctttctcc agctttctct ccagcacggt cttcaggtgg ttgatctggt ggtacacggt 60  
ggccagcagg 70

<210> 14  
<211> 70  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer

<400> 14  
gagctggagt cctggcggaa gatggcgaag atgttctgca gcatctcgta gatggtcaga 60  
gcggcgctct 70

<210> 15  
<211> 70  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer

<400> 15  
cctcggggat gtcgaagttc atcctgtcct tcaggcagta ctccaggcgc ccgttcagct 60  
gccacaggag 70

<210> 16  
<211> 70  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer

<400> 16  
caggaagccg agcaggttgt agctcatcga tagggccgtg gtgctgaagc acaggagcag 60  
ggcgatctgg 70

<210> 17  
<211> 70  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer

<400> 17  
ctgctccaga tcgccctgct cctgtgcttc agcaccacgg ccctatcgat gaagcaccag 60  
caccagcatc 70

<210> 18  
<211> 70  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer

<400> 18  
cactgcttac tggcttatcg aaattaatac gactcactat agggagaccc aagctggcta 60  
gcgtttaaac 70

<210> 19  
<211> 70  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer

<400> 19  
caggaagccg agcaggttgt agctcatctg ttggtgttga tgttggtgct gatgctgggt 60  
ctggtgcttc 70

<210> 20  
<211> 70  
<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 20

agcagggcga tctggagcag gcacttggtg gtcattggtg cgaagcttaa gtttaaagc 60  
tagccagctt 70

<210> 21

<211> 40

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 21

ccgtcagatc ctaggctagc ttattgcggt agtttatcac 40

<210> 22

<211> 32

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 22

gagctcggta ccaagctttt aagagctgta at 32

<210> 23

<211> 77

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 23

gctgaacggg cgcttgagc actgcctgaa ggacaggatg aacttcgaca tccccgagga 60  
aatccgccag ctgcagc 77

<210> 24

<211> 35

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 24

tctccacgcg tacgatggc caggcgcagt ggctg 35

<210> 25

<211> 70

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 25

caccacactg gactagtgga tccttatcag ttgcgcaggt agccgggtcag gcggttgatg 60  
aagtagaagt 70

<210> 26

<211> 31

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 26

catcagcttg ccggtggtgt tgtcctcctt c 31

<210> 27

<211> 31

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 27

gaaggaggac aacaccaccg gcaagctgat g 31

<210> 28

<211> 40

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 28

cacactggac tagtaagctt ttatcagttg cgcaggtagc 40

<210> 29

<211> 47

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 29

gaggagttcg aacttcagc gccagcgcct cctgtggcag ctgaacg 47

<210> 30

<211> 33

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 30

tttaaactgg atccagccac catgaccaac aag

33

<210> 31

<211> 63

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 31

cggccatagt agcgcttcag gtgcagggag ctcacagct tgccggtggt gttgtcctcc 60  
ttc 63

<210> 32

<211> 63

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 32

gaaggaggac aacaccaccg gcaagctgat gagctccctg cacctgaagc gctactatgg 60  
ccg 63

<210> 33

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 33

ggcgtcctcc ttggtgaagt tctgcagctg

30

<210> 34

<211> 39

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 34

atatatccca agcttttatc agttgcgcag gtagccggt

39

<210> 35

<211> 30  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer

<400> 35  
cagctgcaga acttcaccaa ggaggacgcc 30

<210> 36  
<211> 34  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer

<400> 36  
cgcggtacca gccaccatga ccaacaagtg cctg 34

<210> 37  
<211> 34  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer

<400> 37  
cgcggtacca gccaccatga ccaacaagtg cctg 34

<210> 38  
<211> 30  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer

<400> 38  
gtcctccttg gtgaagttga acagctgctt 30

<210> 39  
<211> 39  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer

<400> 39  
atatatccca agcttttatc agttgcgcag gtagccggt 39

<210> 40  
<211> 30



<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer

<400> 40  
aagcagctgt tcaacttcac caaggaggac 30

<210> 41  
<211> 34  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer

<400> 41  
cgcgatcca gccacatga ccaacaagt cctg 34

<210> 42  
<211> 30  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer

<400> 42  
gtcctccttg gtgaagttca ccagctgctt 30

<210> 43  
<211> 30  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer

<400> 43  
aagcagctgg tgaacttcac caaggaggac 30

<210> 44  
<211> 34  
<212> DNA  
<213> Artificial Sequence

<220>  
<223> Description of Artificial Sequence: primer

<400> 44  
cgcgatcca gccacatga ccaacaagt cctg 34

<210> 45  
<211> 30  
<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 45

gtcctccttg gtgaagttcc acagctgctt

30

<210> 46

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 46

aagcagctgt ggaacttcac caaggaggac

30

<210> 47

<211> 34

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 47

cgcggtacca gccaccatga ccaacaagtg cctg

34

<210> 48

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 48

cagcttgccg gtggtgttga actccttctc

30

<210> 49

<211> 30

<212> DNA

<213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: primer

<400> 49

gagaaggagt tcaacaccac cggcaagctg

30

<210> 50

<211> 34

<212> DNA

<213> Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: primer

&lt;400&gt; 50

cgcggtatcca gccaccatga ccaacaagtg cctg

34

&lt;210&gt; 51

&lt;211&gt; 30

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: primer

&lt;400&gt; 51

cagcttgccg gtggtgttca cctccttctc

30

&lt;210&gt; 52

&lt;211&gt; 30

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: primer

&lt;400&gt; 52

gagaaggagg tgaacaccac cggcaagctg

30

**THIS PAGE BLANK (USPTO)**

**This Page is Inserted by IFW Indexing and Scanning  
Operations and is not part of the Official Record**

**BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☒ **BLACK BORDERS**
- ☐ **IMAGE CUT OFF AT TOP, BOTTOM OR SIDES**
- ☐ **FADED TEXT OR DRAWING**
- ☐ **BLURRED OR ILLEGIBLE TEXT OR DRAWING**
- ☐ **SKEWED/SLANTED IMAGES**
- ☐ **COLOR OR BLACK AND WHITE PHOTOGRAPHS**
- ☐ **GRAY SCALE DOCUMENTS**
- ☐ **LINES OR MARKS ON ORIGINAL DOCUMENT**
- ☐ **REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY**
- ☐ **OTHER:** \_\_\_\_\_

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.**

**THIS PAGE BLANK (USPTO)**